Universidade de Aveiro Departamento de Biologia
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Maria de Fátima Tavares de Jesus

EFEITO DA QUÍMICA DA ÁGUA NA FISIOLOGIA E AVALIAÇÃO DA TOXICIDADE EM DAPHNIA

EFFECT OF WATER CHEMISTRY ON THE PHYSIOLOGY AND TOXICITY ASSESSMENT IN DAPHNIA

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor António José Arsénia Nogueira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro e Doutro James Francis Ranville, Professor Associado do Departamento de Química e Geoquímica da Colorado School of Mines, USA

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palavras-chave dureza; alcalinidade; pH; temperatura; metal; *Daphnia magna*; crescimento; reprodução; crescimento populacional; crustáceo; ecossistema aquático

resumo

Todos os sistemas aquáticos estão potencialmente expostos a alterações nos parâmetros da água em consequência de fenómenos ambientais tais como deposição ácida, lixiviação de iões dos solos e alterações climáticas. Dado que os parâmetros químicos da água estão geralmente correlacionados, é pertinente estudar os seus efeitos combinados para o biota aquático. Assim, o principal objetivo desta tese é avaliar a importância ecológica da variação simultânea dos principais parâmetros fisico-químicos da água nos parâmetros de história de vida dos crustáceos tanto na ausência como na presença de metais. Foram estudados os seguintes parâmetros: dureza (0.5 - 3.5 mM), alcalinidade (0.3 - 2.3 mM), pH (5.7 - 9.0) e temperatura (13 - 30ºC). A *Daphnia magna* foi usada como espécie modelo representando os crustáceos aquáticos.

A variação simultânea da dureza e alcalinidade afetou significativamente o crescimento, reprodução e crescimento populacional de *Daphnia*; no entanto, os efeitos da dureza excederam os efeitos da alcalinidade. Pareceu haver interação entre a dureza e alcalinidade na reprodução dos dafnídeos, o que sugere que os efeitos da variação da dureza para *Daphnia* e provavelmente outros crustáceos poderão depender do nível de alcalinidade.

O pH e dureza da água exerceram efeitos combinados para *Daphnia*, sendo os efeitos do pH mais pronunciados do que os efeitos da dureza. A diminuição do pH reduziu a sobrevivência, crescimento, reprodução, taxa de ingestão e crescimento populacional dos dafnídeos. No entanto, os efeitos do pH baixo foram mais adversos a baixa dureza, o que sugere uma interação entre estes parâmetros. Assim, a diminuição do pH em lagos de água mole pode ser um estressor determinante para os crustáceos sensíveis à acidez, ameaçando a sua sobrevivência e, consequentemente, afetando a estrutura das cadeias alimentares aquáticas.

A temperatura e a química da água (dureza e alcalinidade) interagiram entre si nos parâmetros de história de vida de *Daphnia*; no entanto, os efeitos da temperatura excederam os efeitos da química da água. De um modo geral, temperaturas extremas reduziram o crescimento, reprodução e, consequentemente, o crescimento populacional de *Daphnia*. Os efeitos do aumento da temperatura foram mais adversos a baixa dureza e alcalinidade, aumentando a preocupação com os efeitos ecológicos do aquecimento global em águas moles.

A dureza e alcalinidade da água também desempenharam um papel importante na toxicidade aguda e subletal (inibição da ingestão) dos metais para *Daphnia*. A diminuição da dureza aumentou a toxicidade aguda do zinco. Por outro lado, a diminuição da alcalinidade aumentou a toxicidade aguda e subletal do cobre, mas reduziu a toxicidade subletal do zinco, tornando evidente o importante papel da alcalinidade na toxicidade subletal dos metais para *Daphnia*.

Globalmente, os parâmetros fisico-químicos da água parecem interagir entre si, afetando os parâmetros de história de vida e o crescimento populacional de *Daphnia* e também afetam a toxicidade dos metais. Em particular, a baixa dureza agrava os efeitos adversos da diminuição do pH, aumento da temperatura e toxicidade dos metais, o que aumenta a preocupação com os efeitos ecológicos da sua variação simultânea sobre os crustáceos e, portanto, sobre as cadeias alimentares aquáticas.

keywords hardness; alkalinity; pH; temperature; metal; *Daphnia magna*; growth; reproduction; population growth; crustacean; aquatic ecosystem

abstract

All aquatic systems are potentially exposed to changes in the water parameters, as a consequence of environmental current phenomena such as acid deposition, leaching of ions from soils and climate change. Since water chemistry parameters are often correlated, it is pertinent to study their combined effects to aquatic biota. Thus, the main objective of this thesis is to assess the ecological importance of the simultaneous variation of the main water physicochemical parameters to the life-history endpoints of crustaceans either in the absence or presence of metals. The following water parameters were studied: hardness (0.5 - 3.5 mM), alkalinity (0.3 - 2.3 mM), pH (5.7 - 9.0) and temperature (13 - 30ºC). *Daphnia magna* was used as a model species representing aquatic crustaceans.

The simultaneous variation of hardness and alkalinity significantly affected growth, reproduction and population growth of *Daphnia*; however the effects of hardness overruled the effects of alkalinity. There seemed to be an interaction between hardness and alkalinity on daphnids reproduction, suggesting that the effects of varying hardness to *Daphnia* and probably other crustaceans might depend on the alkalinity level.

Water pH and hardness exerted combined effects to *Daphnia*, being the effects of pH more pronounced than the effects of hardness. Decreasing pH reduced survival, growth, reproduction, feeding rate and population growth of the daphnids. However, the effects of low pH were more adverse at low hardness, suggesting an interaction between these parameters. Thus, decreasing pH in soft water lakes might be a major stressor to acid-sensitive crustaceans threatening their survival and, thus, affecting the structure of aquatic food webs.

Temperature and water chemistry (hardness and alkalinity) interacted with each other on the life-history endpoints of *Daphnia*; however, the effects of temperature overruled the effects of water chemistry. In general, extreme temperatures reduced growth, reproduction and, consequently, population growth of *Daphnia*. The effects of increasing temperature were more adverse at low hardness and alkalinity, raising concern about the ecological effects of global warming in soft waters.

Water hardness and alkalinity also played an important role in the acute and sublethal (feeding inhibition) metals toxicity to *Daphnia*. Decreasing hardness raised the acute toxicity of zinc. On the other hand, decreasing alkalinity raised the acute and sublethal toxicity of copper, but reduced the sublethal toxicity of zinc, evidencing the important role of alkalinity in the sublethal toxicity of metals to *Daphnia*.

Overall, water physicochemical parameters seem to interact among each other, affecting life-history endpoints and population growth of *Daphnia* and also affect metals toxicity. In particular, low hardness exacerbates the adverse effects of decreasing pH, increasing temperature and metals toxicity, which raises concern about the ecological effects of their simultaneous variation on crustaceans and, thus, on aquatic food webs.

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Acronym list

AFR: age at first reproduction

BL: body length

BLM: Biotic ligand model

DOC: dissolved organic carbon

dw: dry weight

EC_{50}: median effect concentration (concentration of a chemical that will have 50% of effect on the organisms exposed to it)

EDTA: ethylenediaminetetraacetic acid

FFR: fertility at first reproduction

LC_{50}: median lethal concentration (concentration of a chemical that will cause death to 50% of the organisms exposed to it)

OECD: Organization for Economic Co-operation and Development

r: population growth rate (or intrinsic rate of population increase)

SFR: size at first reproduction

USEPA: United Stated Environment Protection Agency

"Knowledge is not to be sought for the pleasures of the mind, or for contention, or for superiority to others, or for profit, or fame, or power, or any of these inferior things, but for the benefit and use of life."

Sir Francis Bacon

CHAPTER

1

GENERAL INTRODUCTION

1. General introduction

1.1. The model species *Daphnia magna*

As one starts reading this thesis it might wonder: "Why *Daphnia*?". Indeed, *Daphnia* species are among the oldest model systems in biological research (e.g. Lampert 2006; Ebert 2011). Experimental work with *Daphnia* dates back to the 19th century and embraces many research fields, including ecology, ecotoxicology, and heritability (Ebert 2011). In the second half of the 20th century most works with *Daphnia* focused on their role as a component of aquatic ecosystems and their suitability for mechanistic ecological research. Since then, research literature dealing with *Daphnia* has been growing enormously, and nowadays we know more about their ecology than of any other taxon (Lampert 2006; Ebert 2011).

The scientific classification and taxonomy of the genus *Daphnia* is:

Kingdom Animalia
Phylum Arthropoda
Subphylum Crustacea
Class Branchiopoda
Order Cladocera
Family Daphniidae

Daphnia are small planktonic crustaceans, usually less than 5 mm in length. They are commonly called water fleas because of their saltatory swimming style. *Daphnia* have several characteristics that make them suitable as a model organism, namely their wide distribution, numerical abundance and central role in aquatic food webs. *Daphnia* are a widespread inhabitant of standing freshwaters around the world, and are most diverse in North America, Europe and North Asia (Forró, Korovchinsky et al. 2008). In many lakes they are very abundant and can be considered keystone species. The genus *Daphnia* contains a large number of species, making it suitable for ecological and evolutionary studies since the species are adapted to different habitats, whereas the physiological parameters of the different species are similar (Lampert 2006). These planktonic

crustaceans are ecologically relevant as they are often the primary grazers of phytoplankton (as well as bacteria and protozoans), and the primary forage for planktivorous fish. This central position makes them ideal to study interactions between trophic levels within aquatic food webs. For this study it was also determining that *Daphnia* are particularly vulnerable to water chemistry, namely water hardness because of their high Ca demand. Indeed, *Daphnia* are considered the most vulnerable genus to low Ca concentrations and were recently used as a model species to study the effects of Ca decline in aquatic ecosystems (Jeziorski, Yan et al. 2008).

Other advantageous characteristics that favor *Daphnia* as model species include (Lampert 2006; Cairns and Yan 2009):

- they are easy to culture under laboratorial conditions;
- they are large enough to allow individual handling, besides their small size;
- they have a short life-cycle and short reproductive cycles; they mature early, giving birth to neonates before the age of 10 days and, afterwards, every 3 days throughout the remainder of their lives (reference values at 20ºC); under good conditions they may produce more than 50 neonates in a single brood. This allows the production of a large number of individuals in a short time period and is also advantageous in life history and population dynamics experiments;
- they are genetically homogeneous under parthenogenic reproduction, the common type of reproduction under proper laboratorial conditions, which allows to obtain individuals genetically identical;
- juveniles and, to a less extent, adults are transparent allowing the visualization of the functioning of inner organs, making them particularly suitable for morphological and physiological studies;
- they are highly sensitive to toxicants: *Daphnia* are recognized as a sentinel species of lentic freshwater ecosystems (e.g. lakes and ponds), where their decline indicates environmental problems (Dodson and Hanazato 1995). Additionally, *Daphnia* are recommended as standard species in Ecotoxicology (e.g. USEPA 2002; OECD 2008);

- the existence of extensive literature on their physiology, populations dynamics, phenotypic and genotypic variability, and their phylogeny, contributes, for instance, for their suitability for mathematical modeling.

Within the genus *Daphnia*, the species *Daphnia magna* Straus (1820) (Figure 1.1) is one of the most commonly studied. In this study we used this organism as a model species representative of planktonic crustaceans. This species is mainly found in eutrophic ponds, rock pools (Hebert 1978) and shallow lakes (Lampert 1991), which are particularly exposed to varying water chemistry since chemical variations are not buffered by the presence of a large water mass.

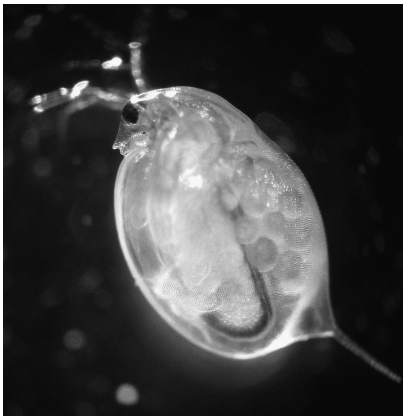


Figure 1.1: *Daphnia magna* Straus (1820) (adult organism).

1.2. Water chemistry

Water is a vital resource to all forms of life. Freshwater is the most common type of water in inland aquatic ecosystems (lakes, ponds, rivers and others) and, thus, is of particular interest.

The chemical properties of freshwater vary widely, both geographically and temporally. The geographic variability of water chemistry is mainly determined by factors such as geology, weathering, climate and land cover/use of the surrounding landscape (Stendera and Johnson 2006). Temporally, water chemistry is affected by dilution, ice formation and

melting (Canfield, Bachmann et al. 1983), evaporation-crystallization (Gibbs 1970), and anthropogenic processes, such as acid deposition and pollution.

Acid deposition is primarily caused by emissions of sulfur dioxide (SO_2) and nitrogen oxides (NO_x) to the atmosphere by, for instance, electric utilities burning fossil fuels. These gases are converted to sulfuric acid and nitric acid in the atmosphere and can be carried by the winds for long distances (Galloway 1995), falling out of the atmosphere either in the dry (acidic gases and particles) or wet form (rain, snow, fog and dew). As a consequence, acid deposition leads to a reduction in pH and alkalinity of the water. Additionally, acid deposition promotes the leaching of base cations from watershed soils, namely calcium (Ca) and magnesium (Mg) (Watmough, Aherne et al. 2005; Ashforth and Yan 2008; Cairns and Yan 2009). Initially, soil leaching of base cations causes an increase in Ca and Mg concentrations in aquatic systems. However, after decades of acid deposition this results in the depletion of Ca and Mg in watershed soils and decreasing inputs to freshwaters (Lawrence, David et al. 1999; Keller, Dixit et al. 2001; Skjelkvåle, Stoddard et al. 2005; Ashforth and Yan 2008).

This phenomenon can be exacerbated by Ca loss from forest biomass harvesting and regrowth after multiple timber harvesting cycles (Huntington, Hooper et al. 2000) and reduced atmospheric Ca deposition (Lawrence, David et al. 1999). Consequently, freshwater systems exposed to acid deposition and large clearances of vegetation have decreased Ca and Mg concentrations. Such trend is observed in many regions of Europe and North America (Skjelkvåle, Stoddard et al. 2005). Jeziorski and coworkers (2008) reported a "widespread threat of calcium decline" in most freshwaters, mainly in soft water lakes affected by acid deposition. For instance, Ca concentrations in the soft water lakes of the south-central Canadian Shield have dropped by over 45% on average since preindustrial times, mostly associated with emissions from the Sudbury area metal smelters (Keller, Dixit et al. 2001).

Water chemistry parameters are closely related. In natural waters there is a significant degree of covariability between alkalinity and hardness (Okland and Okland 1986; Meyer 1999). This is because the main source of alkalinity is usually carbonate rocks (e.g. limestone) that are mostly $CaCO_3$. Thus, usually, soft waters have low alkalinity, whereas hard waters have high alkalinity. However, if the dominant anions in the water are chloride

and sulfate rather than carbonate, this correlation might not be observed. Another common correlation in water chemistry occurs among alkalinity, pH and conductivity (Cowgill and Milazzo 1991b): increasing one of this parameters will cause the others to increase, and vice-versa.

The study of the combined effects of water physicochemical parameters to aquatic biota is pertinent since all aquatic systems are potentially subjected to changes in the water parameters. Among these, the following parameters were addressed in this thesis: hardness, alkalinity, pH and temperature.

1.2.1. Hardness

Total hardness represents the concentration of bivalent ions in the water: calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn) and strontium (Sr). Frequently, it is measured as mg $CaCO_3$/L or mM $CaCO_3$.

As referred previously, declines in Ca and Mg concentrations, and consequently, reduced hardness are widespread in regions recovering from acidification (Lawrence, David et al. 1999; Keller, Dixit et al. 2001; Skjelkvåle, Stoddard et al. 2005), mainly due to depletion of exchangeable base cation pools in lake watersheds (Keller 2009) and clearances of vegetation (Huntington, Hooper et al. 2000).

Hardness is known to dictate the distribution of some aquatic organisms, in particular crustaceans (Waervagen, Rukke et al. 2002; Jeziorski, Yan et al. 2008). Crustaceans have a fully aquatic life-cycle, heavily calcified exoskeleton that is shed regularly, and, in general, lack the ability to store Ca while molting (Porcella, Rixford et al. 1969; Alstad, Skardal et al. 1999; Cairns and Yan 2009), thus, they have large Ca demands. These organisms rely in water Ca concentrations as the primary Ca source, extracting Ca from the water (Wheatly 1999; Hessen, Alstad et al. 2000). The main fraction of this Ca is deposited in the exoskeleton as calcium carbonate (Compère, Jeuniaux et al. 2004) and, whereas some Ca is reabsorbed from the exoskeleton in the pre-molt period (Wheatly 1999), much of it is lost during molting (Greenaway 1985). Therefore, an inadequate supply of Ca could threaten the persistence of crustaceans.

Daphnia are considered the most vulnerable genus to low Ca concentrations which is a reflex of the much higher Ca concentration in *Daphnia* (2 to 8% of dry body weight) than in other zooplanktonic crustaceans (0.2 to 0.4% of dry body weight) (Jeziorski and Yan 2006). Most of the Ca (72%–92% of total body Ca) is located in the exoskeleton (also called carapace) (Muyssen, De Schamphelaere et al. 2009). During molting only 10% of body Ca is reclaimed; the rest is lost both with the shed exuviae (40%) and leaching to the external environment (50%) (Alstad, Skardal et al. 1999).

Hardness, mediated by Ca concentration, is known to affect daphnids survival, growth and reproduction. In general, it is assumed that ambient Ca levels at or below approximately 0.0125 mM are lethal to *Daphnia* (Hessen, Alstad et al. 2000). In laboratory experiments, *D. magna* at 0.0025 mM Ca died within 48 h; at 0.0125 mM Ca they survived until day 10, but suffered 100% mortality within 30 days (Hessen, Alstad et al. 2000).

Growth is mostly affected by low Ca levels immediately after hatching (Hessen, Alstad et al. 2000; Rukke 2002) since juvenile daphnids molt more frequently than adults, thus, have higher Ca demands. In addition, surface to body area ratios are greater in juveniles than in adults resulting in increasing Ca demand for a given carapace thickness (Cairns and Yan 2009). This results in a ‘‘calcium bottleneck’’ in juvenile daphnids and is reflected in the fact that Ca levels that may sustain an adult daphnid may not be adequate for a juvenile (Cairns and Yan 2009). The Ca growth threshold for *Daphnia* ranges from 0.0125 to 0.025 mM Ca (Hessen, Alstad et al. 2000). Below this threshold, reducing Ca concentration reduces body length of 7d-old juveniles (Hessen, Alstad et al. 2000); but above this threshold an opposite trend is observed as reduced Ca concentration causes the body length of juveniles to increase (Winner 1989; Muyssen, De Schamphelaere et al. 2009). Ca levels below Ca growth threshold may, thus, result in reduced body length and length specific dry weight (Hessen, Alstad et al. 2000), with consequences for reproduction and, hence, for the population growth.

Reproduction of *D. magna* is also affected by water Ca concentration. Below 0.25 mM Ca, age-specific egg production is strongly reduced (Hessen, Alstad et al. 2000). This might be related to the reduced growth rates but could also be a consequence of the higher energetic costs associated with Ca uptake (Hessen, Alstad et al. 2000). Low Ca concentrations

increase age at first reproduction (Cowgill and Milazzo 1991a; Hessen, Alstad et al. 2000; Cairns and Yan 2009) and decrease total reproduction (Cowgill and Milazzo 1991a).
Therefore, chronically low Ca levels may lead to reduced abundance of *Daphnia* and, thus, reduced population size (Tessier and Horwitz 1990; Jeziorski, Yan et al. 2008), which can have strong effects on aquatic food webs (Jeziorski et al. 2008). Moreover, low Ca can also increase the susceptibility of aquatic organisms to other abiotic factors, such as temperature (Ashforth and Yan 2008), acidity (Hooper, Connon et al. 2008), UV radiation (Hessen and Rukke 2000) as well as to toxic metals (e.g. de Schamphelaere and Janssen 2002b).

1.2.2. Alkalinity

Water alkalinity may be defined as its capacity to neutralize acids, which is an expression of buffering capacity. Alkalinity is mainly due to the presence of hydroxides, carbonates and bicarbonates. However, other anions, such as borates, silicates, and phosphates also contribute to alkalinity. Like hardness, it is commonly measured as mg $CaCO_3$/L or mM $CaCO_3$.
Alkalinity is important for aquatic life because it protects or buffers against rapid pH changes. Higher alkalinity levels in surface waters will protect aquatic life from fluctuations in pH caused by acid deposition or acid contamination. However, little is known about the effects of alkalinity to aquatic organisms. Indeed, the effects of alkalinity are often expressed in function of pH, since these water chemistry parameters are highly correlated (Cowgill and Milazzo 1991b).
Low alkalinity levels have no significant effects on survival and reproduction of *Daphnia*, but high alkalinity levels might negatively affect these endpoints. According to Cowgill and Milazzo (1991b; 1991a) high alkalinity levels caused a slight delay in sexual maturity, reduced cumulative fertility and, thus, reduced intrinsic rate of population growth. Levels above 3.5 mM as $CaCO_3$ were reported to cause mortality of *D. magna* (Cowgill and Milazzo 1991b). These authors (1991b; 1991a) demonstrated that the toxicity of increased alkalinity was not due to increased Na concentration (alkalinity was manipulated by adding different amounts of $NaHCO_3$) or to high pH, but to alkalinity *per se*.

The effects of alkalinity on crustaceans growth, particularly daphnids, remain fairly unstudied. Though, Zhao and coworkers (2007) showed that growth of *Daphniopsis tibetana* reared at alkalinity 4.8 to 18.9 mM did not significantly differ, which might suggest a reduced effect of alkalinity on daphnids growth.

Alkalinity can also affect the sensitivity of aquatic organisms to some metals, namely copper (Shaner and Knight 1985) and mercury (Spry and Wiener 1991).

1.2.3. pH

Water pH is a measure of its acidity, and is given as: $pH=-\log[H^+]$, where $[H^+]$ represents the concentration of protons (M), and can vary between 1 and 14.

Usually, pH of natural waters varies between 6.5 and 8.5, although values out of this range can be found for instance, in Portugal. Due to the geochemistry of soils and rocks, waters in the North of Portugal tend to be acidic and waters in the South tend to be alkaline. For example, the natural mineral water from Luso (a village in the north-center of Portugal) has a mean pH value 5.7 (chemical analysis LNEG, bulletin 02/H/2011) whereas the mineral water from Serra de Monchique (South of Portugal) has a mean pH value 9.3 (chemical analysis I.G.M., 19-10-2004).

Besides its geographical variability, water pH also varies temporally, which can be related, for instance, with water acidification. Water acidification has been recognized as a major environmental problem in many parts of Europe and North America for several decades, and is a consequence of both natural processes and anthropogenic activities. Natural processes leading to water acidification include spring acid episodes (Laudon, Westling et al. 2000), acid rock drainage (Ezoe, Lin et al. 2002; Eppinger, Briggs et al. 2007) and volcanism (Ezoe, Lin et al. 2002). Anthropogenic activities causing water acidification include agriculture (Kurvits and Marta 1998), dredging of sediments (Cappuyns, Swennen et al. 2006), mining-related processes such as acid mine drainage and re-cultivation and flooding of former mining pits (Bortnikova, Smolyakov et al. 2001) and fossil fuels burning which affect water bodies through acid deposition (Galloway 1995). Among these processes, acid deposition emerged as a major international concern during the 1980s. Although acid deposition effects on lakes had been identified much earlier, it was during

this decade that scientific, public, and political recognition of the problem came to the forefront. As a consequence, emissions control programs[1] were developed which contributed to reduce acid deposition, as shown in recent investigations: the vast majority of the formerly acidified lakes in Europe and North America are now facing an increase in water pH (Skjelkvåle, Stoddard et al. 2005). However, the recovery from acidification has been accompanied by decreasing concentrations of sulfates, calcium and magnesium, and increased concentration of dissolved organic carbon (DOC) (Keller, Dixit et al. 2001; Skjelkvåle, Stoddard et al. 2005; Keller 2009). Declines in calcium concentration are of particular concern because of the implications for aquatic biota, as referred previously: low Ca not only affects crustaceans abundance and distribution, but also increases their susceptibility to other abiotic factors.

Water acidification affects the distribution and structure of zooplankton communities through several mechanisms, as synthesized by Weber and Pirow (2009). These mechanisms include a differential physiological sensitivity to acid stress (Locke 1991; Havens, Yan et al. 1993), a differential sensitivity to toxic metal species which become more soluble under acidic conditions (Lawrence and Holoka 1987; Havens 1990), and also altered biotic interactions due to the effects of reduced pH on phytoplankton and planktivore communities (Locke and Sprules 2000).

The physiological sensitivity of aquatic animals to acidic conditions is associated with increased membrane permeability, including processes such as swelling of the heart muscle, rapid fading of hemoglobin, and a net loss of Na and Cl (Havas, Hutchinson et al. 1984). In an external environment enriched in H^+, organisms face a continuous diffusive loss of ions and gain of water. Under these conditions, most organisms have to minimize their whole-body permeability to water and/or ions and also to improve uptake mechanisms for Na and Cl to maintain a steady-state ion balance (Mantel and Farmer 1983). For instance, because of their relatively low sodium permeability, aquatic insects are acid-tolerant species.

[1] The international UNECE monitoring program ICPWaters (International Cooperative Programme on Assessment and Monitoring of Rivers and Lakes) is an example of an emission control program.

The majority of the *Daphnia* species are considered acid-sensitive (Locke 1991; Havens, Yan et al. 1993). The disruption of Na metabolism is correlated with the net loss of Na^+ due to both accelerated rate of Na^+ loss and reduced rate of Na^+ uptake, a process that is additionally influenced by the ambient calcium concentration (Glover and Wood 2005). At high Ca concentrations, lowering water pH severely inhibits sodium influx, but at very low Ca concentrations an opposite pattern is observed (Glover and Wood 2005). Decreasing pH affects feeding rate, heart rate, respiration rate and, thus survival, longevity, growth, reproduction and intrinsic rate of population growth of *Daphnia* (Kring and O'Brien 1976; Alibone and Fair 1981; Walton, Compton et al. 1982; Havas 1985; Locke and Sprules 2000; Weber and Pirow 2009). For this reason, *Daphnia* are not usually found at pH values below 5.8 (Almer, Dickson et al. 1974).

1.3. Temperature

Public awareness of global warming has raised concerns about its impact on the Earth's ecosystems extending all the way to human societies. This concern is justified as about 99.9% of species on Earth are ectothermic, that is, they rely primarily on external sources for their body heat (Kammenga, Doroszuk et al. 2007). This means that the body temperature is almost exactly the same as that of the environment. Concordantly, in the aquatic environment most of the organisms, including fish and invertebrates, are ectotherms. Indeed, with the exception of marine mammals, birds, and tuna fish, essentially all aquatic organisms are ectothermic (Cairns, Heath et al. 1975).

Under climate change, temperature anomalies, such as heat waves, are expected to occur more frequently (Huber, Adrian et al. 2010) and can have serious repercussions in aquatic ecosystems. The effects of temperature are particularly pronounced in species living in shallow-water ecosystems (ponds, pools and marshes), since temperature variations are not buffered by the presence of a large water mass (Gordo, Lubian et al. 1994). In these ecosystems, water temperature can vary widely both daily and seasonally. Daily variations can occur rapidly as a result of the succession day-night. Additionally, zooplankton with diurnal vertical migration behavior, such as *Daphnia*, are also exposed to rapid temperature changes during vertical migration (Orcutt and Porter 1983). Seasonal changes, in other

hand, occur slowly and are due to both the succession of seasons and to global climate change (Gordo, Lubian et al. 1994; Pörtner, Bennett et al. 2006).

The likely oxygen shortage at extreme temperatures makes aquatic organisms particularly vulnerable to the effects of temperature changes namely global warming. An increase in water temperature increases metabolic activity and, thus, oxygen demand of aquatic organisms (Mourelatos and Lacroix 1990). However, when exposed to the increase of water temperature, aquatic organisms also have to face the subsequent decrease of oxygen solubility in the water. In other hand, at low temperatures, although oxygen demand is reduced, the remaining energy metabolism may not be sufficiently supplied with oxygen because of the cessation of ventilation and perfusion and the muscular activity necessary for these processes (Zeis, Maurer et al. 2004). Oxygen limitation contributes to oxidative stress that affects cellular mechanisms.

Temperature is a critically important abiotic factor for ectotherms because it affects all biological processes (Pörtner, Bennett et al. 2006). According to their thermal tolerance, ectotherms may be classified as stenotherms (narrow thermal tolerance window) or eurytherms (wide thermal tolerance window). The thermal tolerance window usually has a bell shape: the optimum curve (Kersting 1978). The optimum temperature is characterized by maximal somatic growth and reproduction. The suboptimal temperature ranges are characterized by decreasing performance and extend to both low and high end of the thermal tolerance window, i.e. they extend to the critical temperatures. Exceeding these limits will cause physiological processes to break down and will lead to death (Goss and Bunting 1976). In general, within the temperature range at which an organism can grow and reproduce, the general trend is for an increment in temperature to cause metabolic and growth rates to increase, and body length and longevity to decrease (Mourelatos and Lacroix 1990; McKee 1995; Atkinson and Sibly 1997; Gillooly, Brown et al. 2001). Concerning reproduction, when reared at higher temperatures, ectotherms reproduce earlier at a smaller size. This is known as the temperature–size rule (Kammenga, Doroszuk et al. 2007) and nearly 90% of ectothermic species, including *Daphnia*, follow this rule (Atkinson 1994).

In *Daphnia*, temperature affects respiration rate, ventilatory frequency (thoracic limb movements) and heart rate (Koh, Hallam et al. 1997; Lamkemeyer, Zeis et al. 2003; Paul,

Lamkemeyer et al. 2004). Temperature also affects feeding rate (Mourelatos and Lacroix 1990; Koh, Hallam et al. 1997), thus influencing growth and development (Lei and Armitage 1980; Goss and Bunting 1983) and reproduction (Goss and Bunting 1983; Giebelhausen and Lampert 2001). The duration of the juvenile period is also affected, which will be reflected as effects on age at first reproduction and, consequently, on the body length and number of eggs in the first reproduction (McKee and Ebert 1996; Giebelhausen and Lampert 2001). The duration of adult instars is also affected due to the effects of temperature on the duration of embryonic development (Goss and Bunting 1983). Moreover, temperature affects longevity (Lei and Armitage 1980; Armitage and Landau 1982) and survival (Zeis, Maurer et al. 2004). The response curves of these mechanisms have different shapes, as depicted in Figure 1.2.

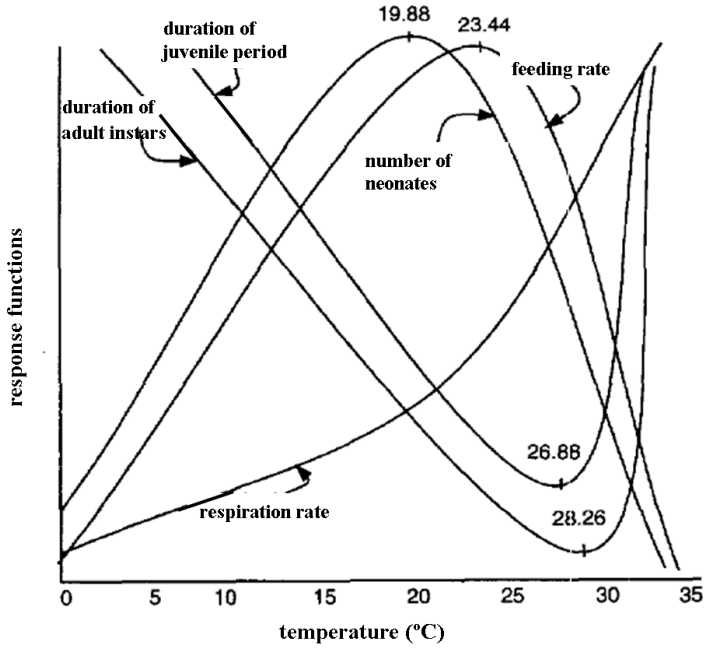


Figure 1.2: Temperature response functions of *Daphnia magna*. Values marked on curves refer to the respective optimal temperatures (adapted from Koh, Hallam et al. 1997)

The response of crustaceans to elevated temperatures depends not only on the magnitude of the temperature change but also on its seasonal timing. For instance, juvenile daphnids are more tolerant to temperature than adults (Hall and Burns 2002). Additionally, certain

crustacean life cycle events are particularly sensitive to temperature and photoperiod, such as emergence from resting eggs and egg development (Vijverberg 1980; Gyllström and Hansson 2004).

At the cellular level, the most frequently referred alterations caused by high temperatures include increasing perturbation of membrane and protein functions, particularly impairment of enzyme kinetics, denaturation of proteins, depletion of resources and damage by oxygen radicals (Zeis, Maurer et al. 2004). To face these potentially lethal alterations, organisms increase the production of stress proteins (e.g., heat shock proteins), which contribute to the stabilization and reestablishment of the proper functioning of partly denatured proteins. However, the synthesis of stress proteins requires additional energy and may raise maintenance costs (Ashforth and Yan 2008), which is complicated by the reduced availability of oxygen in the water. Under these conditions, when convective oxygen supply approaches maximum values, a widening gap between oxygen supply and energy demand occurs which forces organisms to recur to anaerobic energy metabolism (Zeis, Maurer et al. 2004). However, at more extreme temperatures only time limited survival is supported by anaerobic metabolism (Pörtner 2002). Similarly, at very low temperatures the aerobic capacity of mitochondria may become limiting for ventilation and circulation resulting in the cessation of aerobic metabolisms and transition to the anaerobic mode of mitochondrial metabolism and results in progressive insufficiency of cellular energy levels (Pörtner 2002).

In natural ecosystems organisms respond to changes in temperature at a wide range of levels of organization, from molecular to physiological and behavioral (Pörtner, Bennett et al. 2006). In consequence, the thermal tolerance window may be altered by acclimation on a life-time scale or by adaptation during evolutionary periods (Zeis, Maurer et al. 2004). Most ectotherms, if exposed for a long period of time to unfavorable (but not lethal) temperatures, have the ability to adapt to the new temperature conditions, a process known as acclimation (e.g. Johnston and Dunn 1987; McKee 1995). The acclimation process can be described as an adjustment of oxygen supply and energy provision to existing temperatures (Zeis, Maurer et al. 2004) which allows an individual to stabilize physiological rates and, thus, respond to the temperature changes in optimal way (McKee 1995). The mechanisms underlying acclimation include alteration of mitochondrial aerobic capacity (Pörtner 2002) and changes of protein quantity or quality (expression of orthologs)

more suitable for the new temperature conditions (Somero 2004). The acclimation process has been largely studied in *Daphnia*. Temperature acclimation in *D. magna* is related to oxygen transport processes which, in turn, are related to changes in quantity and quality of hemoglobin (Lamkemeyer, Zeis et al. 2003), or, in other words, temperature acclimation in *D. magna* implies hemoglobin induction by hypoxia (Zeis, Maurer et al. 2004). Laboratorial studies on the time course of temperature acclimation of *Daphnia* showed that the major processes are completed in 2 weeks, but major changes occur in the first 3–5 days (Zeis, Maurer et al. 2004; Seidl, Pirow et al. 2005). Acclimation to a different temperature implies changing the thermal tolerance window, so the acclimation temperature becomes the optimum temperature. For instance, *D. magna* have higher feeding rates and preferred temperatures at the acclimation temperature (McKee 1995; Lamkemeyer, Zeis et al. 2003). Temperature acclimation also plays a key role in the effects of high temperatures in *Daphnia* (Paul, Lamkemeyer et al. 2004): warm-acclimated *Daphnia* have improved capacity to survive heat, as, for instance, higher quantities of heat-shock proteins (Feder and Hofmann 1999).

Concerning evolutionary adaptation, *Daphnia* populations can have a clonal structure which allows their persistence over a wide temperature range due to a broad temperature reaction norm or to the succession of clones with different reaction norms during the seasons (Carvalho 1987; Mitchell and Lampert 2000; Chopelet, Blier et al. 2008).

In ecosystems, organisms are exposed to varying stressors, which can interact among each other generating non-additive effects that are difficult to predict based on single-stressor studies (Christensen, Graham et al. 2006). These interactions can have different effects depending on the stressors involved and on the organisms. For instance, Christensen and coworkers (2006) found that interactions among warming, drought, and acidification exerted significant synergistic and antagonistic effects on consumers and producers, respectively. Concerning *Daphnia*, temperature affects their response to other natural stressors, such as photoperiod (Armitage and Landau 1982; Korpelainen 1986), salinity (Hall and Burns 2002), oxygen levels (Lamkemeyer, Zeis et al. 2003; Paul, Lamkemeyer et al. 2004), food levels (Orcutt and Porter 1984; Giebelhausen and Lampert 2001) and Ca concentration (Ashforth and Yan 2008). Temperature also affects the susceptibility of

Daphnia to anthropogenic stressors, such as aquatic pollutants (e.g. Boeckman and Bidwell 2006).

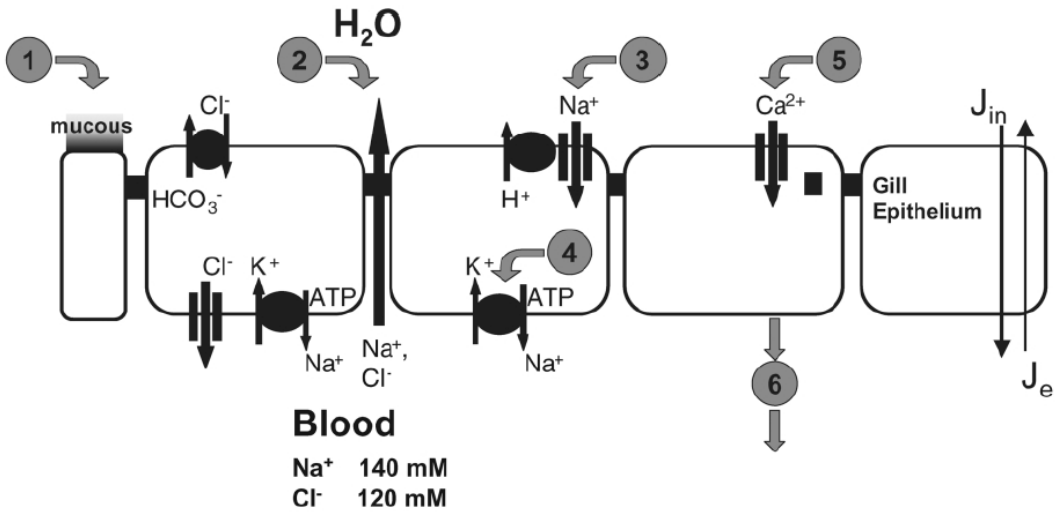
1.4. Water chemistry and metals toxicity

Metal contamination in aquatic systems is one of the most ubiquitous and persistent environmental problems, caused by both natural and anthropogenic processes**.** Metals occur naturally in the Earth's crust and their distribution is primarily controlled by the geochemistry of the underlying and local rocks. As rocks erode and weather to form soils and sediments, metals are released and can enter the aquatic systems (Garrett 2000). Moreover, natural processes such as the escape of gases and fluids along major fractures in the Earth's crust, and volcanic activity, can provide significant sources of metals to surface environments (Garrett 2000). Currently, natural inputs of metals to aquatic systems are exceeded by anthropogenic inputs (Rai 2008). Anthropogenic sources include mining and smelting of metalliferous ores, burning of fossil fuels with subsequent atmospheric deposition, municipal wastewater and agriculture (Jeffries and Snyder 1981; Chino, Moriyama et al. 1991; Baker 1992; Milu, Leroy et al. 2002; Nicholson, Smith et al. 2003). The metal load from domestic wastewater and sewage alone (Chino, Moriyama et al. 1991) ensures that this will be a continuing problem for science and humankind (Rai 2008). In addition, contrarily to other toxicants, metals are inherently persistent as they are neither created nor destroyed by anthropogenic or biological processes, although they may undergo various speciation changes in abiotic and biotic systems (Peijnenburg and Vijver 2007). High metal levels in surface water may pose a health risk not only to the environment, but also to humans.

In this study we focused on the metals copper (Cu) and zinc (Zn). Both these metals are trace elements found in the Earth's crust and, thus, naturally occurring elements generally present in surface waters. These metals are essential trace metals to living organisms, but may become toxic at high concentrations. Very high concentrations of these metals can be found in, for instance, superficial waters nearby mining areas. Concentrations as high as 15.92 µM Cu and 76.93 µM Zn were found in stream waters nearby a copper mine in

Romania (Milu, Leroy et al. 2002). In Bolivia, concentrations of 45.32 µM Cu and 351.48 µM Zn were found in waters of the river Tarapaya, which is strongly affected by mine tailings (Smolders, Lock et al. 2003). Moreover, high concentrations of metals can also be found in drinking water from public water supplies, as illustrated by the copper value of 7.87 µM or more found in 10.3% of households in southern Lower Saxony, Germany (Zietz, De Vergara et al. 2003).

The physiological mechanisms underlying metal toxicity to aquatic species, particularly fish, have been the subject of numerous studies (see reviews by Paquin, Gorsuch et al. 2002; Rainbow 2002; Luoma and Rainbow 2005). As summarized by Paquin and coworkers (2002), and illustrated in Figure 1.3, the mechanisms of toxicity for most metals can generally be divided into three categories: monovalent metals (e.g. Ag^{+} and Cu^{+}) affect Na transport; divalent metals (e.g. Cd^{2+} and Zn^{2+}) disrupt Ca metabolism due to competition for binding sites; and some other metals (e.g. Pb^{2+}, Hg^{2+}) cross the gills and act centrally (Niyogi and Wood 2004). Note that Cu, although it mainly exists as a divalent metal (Cu^{2+}) in water, it is probably reduced to monovalent Cu^{+} before being transported across biological membranes (Paquin, Gorsuch et al. 2002).



1- adsorption of metals resulting in gas transfer problems (increased mucous/diffusion distance)
2- metals that affect tight junctions altering efflux/ion loss (J_e)
3- metals that block Na^+ uptake (J_{in}) directly at transport site
4- metals that block Na^+ uptake (J_{in}) indirectly by inhibiting Na^+/K^+ ATPase
5- metals that block Ca^{2+} uptake (J_{in}) directly at transport site
6- metals that are absorbed and affect the fish centrally

Figure 1.3: Schematic diagram of the main mechanisms of metal interaction at the fish gill leading to adverse effects (Paquin, Gorsuch et al. 2002)

1.4.1. Biotic ligand model

Metals toxicity is known to be dependent on temperature and water chemistry parameters, namely hardness, pH, DOC and concentration of major ions. Researchers have developed a model to evaluate the effect of water chemistry on the speciation and bioavailability of metals in aquatic systems and, thus, to predict metals toxicity: the biotic ligand model (BLM) which conceptual framework was originally developed by Di Toro and coworkers (2001) and further refined by other researchers. The BLM framework combines of tree mathematical models: the gill surface interaction model (Pagenkopf 1983), the free ion activity model (Morel and Hering 1993), and the geochemical speciation model Windermere humic aqueous model (WHAM) 5.0 (Tipping 1994).

The BLM approach has gained widespread interest amongst the scientific and regulatory communities because of its potential to be used in the development of water quality criteria and aquatic risk assessments for metals (Paquin, Gorsuch et al. 2002). In 2007 the U.S. EPA updated its freshwater copper criteria by incorporating the use of the BLM in the criteria derivation procedures (USEPA 2007). The BLMs are specie-specific and metal-specific, resulting in the development of specific models for various organisms and metals, as discussed below.

The key assumption of the BLM is that metal toxicity occurs as a consequence of free metal ions reacting with binding sites (biochemical receptors) at the organism-water interface. These binding sites, called “sites of action of toxicity” or “biotic ligands” can be either physiologically active sites (leading to a direct biological response) or transport sites (leading to metal transport into the cells followed by an indirect biological response) (De Schamphelaere and Janssen 2002a). For instance, in the case of fish, the biotic ligands have been identified with gill sites involved in ion exchange regulation (Pagenkopf 1983; Playle, Gensemer et al. 1992). The binding of metal ions to the biotic ligand is represented as the formation of a metal-biotic ligand complex. However, the formation of the metal-biotic

ligand complex is not merely proportional to the metal concentration in the environment, but varies according to the organism, the metal, and the exposure environment (water chemistry). In particular, the water chemistry effect is related to chemical mechanisms such as competition, chemical binding, complexation, absorption, adsorption and precipitation of the metal (Peijnenburg and Vijver 2007). The level of accumulation of the metal at the site of action, i.e., the concentration of the metal-biotic ligand complex determines the magnitude of the toxicological response (De Schamphelaere and Janssen 2002a; Paquin, Gorsuch et al. 2002). The BLM assumes that the relation between the observed effect and the metal fraction bound to the biotic ligand BL is not altered by the physicochemical parameters of the test medium (Heijerick, De Schamphelaere et al. 2002). Another assumption of the BLM is that the chemistry of the system is at equilibrium (Paquin, Gorsuch et al. 2002). Figure 1.4 illustrates the BLM concept adapted to *Daphnia*, the test organism used in this thesis.

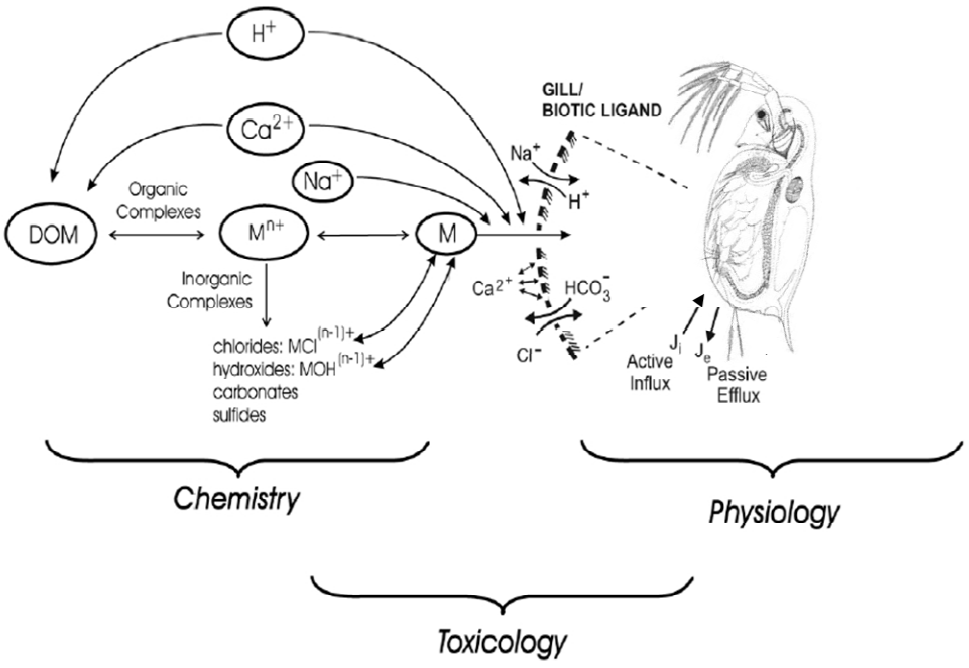


Figure 1.4: Schematic representation of the biotic ligand model for *Daphnia* (adapted from Paquin, Gorsuch et al. 2002)

The exposure environment determines the distribution of the metal among various fractions and, thus, the relative concentrations of chemical species that may have different uptake

rates; hence, the exposure environment controls the bioavailability of the metal. Figure 1.5 illustrates the various metal fractions that can be found in water, soil or sediment.

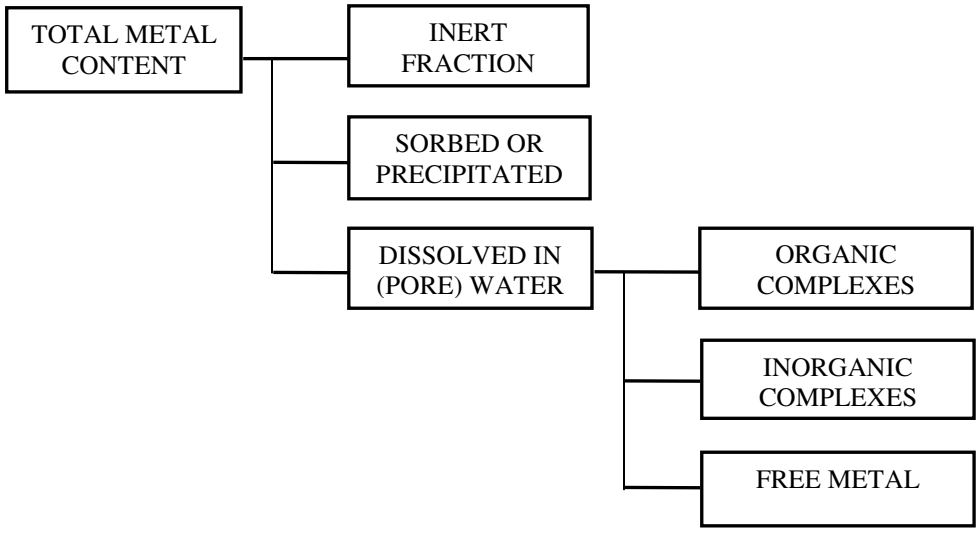


Figure 1.5: Schematic overview of the metal fractions present in water, soil or sediment matrices (adapted from Peijnenburg and Jager 2003)

Metals are commonly associated with ligands (i.e., complexing chemicals with which metals interact, including ions, molecules, or molecular groups), forming either organic or inorganic complexes. Organic complexes are usually formed with DOC, which consists of small organic molecules (e.g. amino acids and sugars) or bigger fulvic and humic acid molecules. Additionally, strong complexes may be formed with organic chelating agents like EDTA (ethylenediaminetetraacetic acid) and NTA (nitrilotriacetic acid). Inorganic complexes can be formed with chemical species such as carbonate, sulfate, hydroxide and chloride. However, complex formation is strongly metal specific (Peijnenburg and Jager 2003): for instance, Cu is more strongly bound to DOC than zinc, and hence copper is in general less available for uptake by aquatic organisms (Peijnenburg and Jager 2003).

As referred previously, metals bioavailability is affected by exposure conditions and determines metals toxicity. However, it should not be presumed that all the effects of water chemistry parameters on metals toxicity reflect effects on bioavailability. For instance, ions

such as Ca and Na play important roles in iono- and osmoregulatory processes (Glover and Wood 2005).

Initially, BLMs were developed mainly to predict acute toxicity of Cu and silver (Ag) to daphnids and fish. However, given the widespread interest of the BLM approach among scientific and regulatory communities, scientists have developed BLMs for several other metals (including Zn, Ni, Cd, etc.) and also for several organisms, namely algae, plants, crustaceans, fish, soil organisms and rotifers, among others (p.e. De Schamphelaere, Lofts et al. 2005; De Schamphelaere, Heijerick et al. 2006; Lock, De Schamphelaere et al. 2006; Lock, Van Eeckhout et al. 2007; Deleebeeck, De Laender et al. 2009). Furthermore, research on BLMs was extended to chronic (sublethal) toxicity to predict EC_{50} and/or NOEC (no observed effect concentration) for effects on reproduction. However, to the best of our knowledge no model has been developed to predict metals toxicity to sublethal endpoints such as feeding rates.

The feeding activity of *D. magna* constitutes a sensitive indicator of toxicity and is an ecologically relevant endpoint (McWilliam and Baird 2002). Food intake determines energy acquisition and further allocation, affecting developmental rate, growth rate, fecundity, and survival of individuals (Nogueira, Baird et al. 2004), thus determining population structure and dynamics.

Determining metal toxicity (percent response) to an organism using the BLM requires that metal speciation, the activity of each cation in solution, and the stability constant for each cation to the biotic ligand(s) for that species are known (Heijerick, De Schamphelaere et al. 2002). However, knowing all these parameters to develop a BLM requires a considerable amount of work and is time consuming. Here, we hypothesized that metal toxicity might be described in a simpler way, based only on hardness and alkalinity as integrative water chemistry parameters.

1.4.2. Copper toxicity

In natural oxygenated waters, “free” copper (Cu) can be found as cupric ion (Cu^{2+}) weakly associated with water molecules ($Cu.nH_2O^{2+}$), but this species is usually a small fraction of dissolved copper. Most dissolved copper is associated with organic ligands, forming strong

complexes (Xue and Sigg 1993). Cu can also be associated with other ligands, namely hydroxides and carbonates (USEPA 2007). Additionally, Cu can be adsorbed to or incorporated into suspended particles (USEPA 2007). More information on copper speciation in freshwaters can be found in previous studies (Kramer, Allen et al. 1997; Bryan, Tipping et al. 2002; Smith, Bell et al. 2002).

Acute Cu toxicity to freshwater invertebrates and vertebrates is due to the inhibition of Na uptake and/or an increase of Na efflux (Grosell and Wood 2002; Taylor, Wood et al. 2003), related to the reduction of Na-K ATPase activity (Playle, Dixon et al. 1993a; Brooks and Mills 2003) at an apical Na channel (Grosell and Wood 2002). This apical Na channel is accepted to be the binding site (or biotic ligand) for Cu (Paquin, Gorsuch et al. 2002).

Cu toxicity to freshwater aquatic organisms varies markedly with water physico-chemical parameters, mainly dissolved organic matter and pH (De Schamphelaere and Janssen 2004c; Park, Jo et al. 2009), but also with inorganic ions, including those related to hardness and alkalinity (see reviews by Allen and Hansen 1996; Paquin, Gorsuch et al. 2002).

The amount of dissolved organic matter, namely DOC, has been referred as the main parameter determining Cu toxicity (De Schamphelaere, Heijerick et al. 2002; De Schamphelaere, Vasconcelos et al. 2004; Park, Jo et al. 2009). Previous studies reported that both acute and chronic Cu toxicity to fish and crustacean species decreases with increasing DOC (e.g. Erickson, Benoit et al. 1996; De Schamphelaere and Janssen 2004c; De Schamphelaere, Vasconcelos et al. 2004; Hyne, Pablo et al. 2005). This is likely related to the complexation of Cu by DOC, which decreases the bioavailability of the metal (Playle, Dixon et al. 1993b), and thus, its toxicity. Additionally, different sources of organic matter have been shown to influence Cu toxicity to varying degrees but to a less extent than DOC concentration (De Schamphelaere, Vasconcelos et al. 2004; Ryan, Van Genderen et al. 2004).

As referred previously, pH is also a main parameter affecting acute Cu toxicity. In general, increasing pH reduces Cu acute toxicity (e.g. Schubauer-Berigan, Dierkes et al. 1993; Erickson, Benoit et al. 1996; Park, Jo et al. 2009) and chronic toxicity (Welsh, Skidmore et al. 1993; De Schamphelaere and Janssen 2004c) to fish and crustacean species. Indeed, pH

is often recognized as a master variable affecting metal speciation (Peijnenburg and Jager 2003), as illustrated in Figure 1.6.

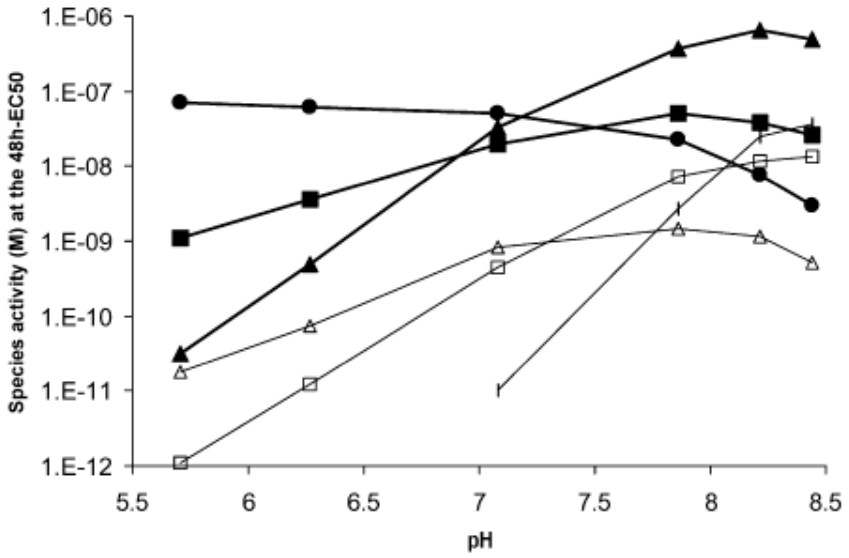


Figure 1.6: Activity of the Cu species at the 48-h EC_{50} level (varying between 3.35 and 6.89 µM Cu) for several pH values (referring to a particular set of tests). Bold lines denote the most important species and thin lines denote less important species. The following Cu species are presented: Cu^{2+} (●), $CuOH^{+}$ (■), $CuCO_3$ (▲), $Cu(OH)_2$ (□), $CuHCO_3$ (△) and $Cu(CO_3)_2$ (|) (De Schamphelaere, Heijerick et al. 2002).

The effect of pH on Cu toxicity is, thus, not concordant with the increased proton competition with Cu^{2+} at low pH (Santore, Di Toro et al. 2001), as this would result in decreased toxicity with decreasing pH. The effect of pH on Cu toxicity is likely due to two factors: in one hand, metals tend to be more soluble (and thus, more bioavailable) as pH decreases (Peijnenburg and Jager 2003), with Cu^{2+} being the major Cu species under acidic conditions (De Schamphelaere, Heijerick et al. 2002; Komjarova and Blust 2009); on the other hand, in the presence of dissolved organic matter (DOM), decreasing pH causes a reduction of the complexation Cu-DOM, which results in increasing of free metal ion (Lu and Allen 2002).

As for other metals, the effect of water hardness on Cu toxicity has been widely studied. The acute Cu toxicity to fish and crustaceans decreases with increasing hardness (e.g. Erickson, Benoit et al. 1996; De Schamphelaere and Janssen 2002a; Naddy, Stern et al. 2003). The antagonistic effects of Ca^{2+} and Mg^{2+} to Cu toxicity are usually attributed to

increased competition for binding sites at the site(s) of action and/or lowering of the ionic activity of the metal (De Schamphelaere and Janssen 2002a). In particular for Ca, another mechanism against Cu toxicity is suggested (Komjarova and Blust 2009): since Na^+ uptake is inhibited by Ca^{2+} (Glover and Wood 2005) this will indirectly reduce Cu uptake and, thus, its toxicity. Additional benefits of increased Ca are also likely to occur due to decrease of the Na efflux, since Ca acts as a stabilizer of tight junctions in epithelial membranes, reducing diffusive loss of ions such as Na and Cl via paracellular pathways (Paquin, Gorsuch et al. 2002). However, other studies report that hardness has little or no effect on acute Cu toxicity to aquatic organisms (Hyne, Pablo et al. 2005; Markich, Batley et al. 2005). Additionally, in the presence of organic matter, the chronic Cu toxicity to crustaceans is also not affected by hardness (Winner 1985; De Schamphelaere and Janssen 2004c). Winner (1985) argued that, although Ca and Mg may not have a direct effect on chronic Cu toxicity, they may have a small indirect effect through competition with Cu for binding sites on the organic matter. This is concordant with the findings of Komjarova and coworkers (2009) which reported that Ca (0.1 to 2.5 mM) had no effect on Cu uptake by *D. magna*.

Some studies suggest that the ratio between Ca and Mg may be more important than their absolute concentrations. However, the effect of the ratio Ca:Mg seems to be variable, depending on several aspects, namely the test organism, the hardness level and the metal. For example, several studies reported that hardness consisting mainly of Ca (Ca:Mg molar ratios $\geq$1) is protective of both fish (Erickson, Benoit et al. 1996; Welsh, Lipton et al. 2000; Naddy, Stubblefield et al. 2002) and crustaceans (De Schamphelaere and Janssen 2002a; Naddy, Stubblefield et al. 2002), whereas hardness consisting mainly of Mg (Ca:Mg molar ratios of $\leq$1) has only been shown to be important for invertebrates (De Schamphelaere and Janssen 2002a; Gensemer, Naddy et al. 2002; Naddy, Stubblefield et al. 2002). Also, it was shown that Cu toxicity increased at higher Ca:Mg ratios for *D. magna*, but, in opposition, it did not vary under different Ca:Mg ratios for *Ceriodaphnia dubia* (Naddy, Stubblefield et al. 2002). Concerning the dependency on the hardness level, a previous study found that although Cu toxicity to *D. magna* increased at higher Ca:Mg ratios for a hardness level 1.8mM, it did not vary under different Ca:Mg ratios at hardness 0.9mM (Naddy, Stubblefield et al. 2002). Additionally, the ratio Ca:Mg is likely to be affected by the metal,

since it was shown that Ca and Mg have different protective effects against Cu and Zn toxicity. For instance, the effect of Mg was shown to be about 1/3 or 1/4 of the effect of Ca against Zn toxicity to *Daphnia* (Heijerick, De Schamphelaere et al. 2005; Clifford and McGeer 2009), whereas Mg was found to be equally or more protective than Ca against Cu toxicity to the same species (De Schamphelaere and Janssen 2002a; Naddy, Stubblefield et al. 2002).

Alkalinity has also been found to affect Cu toxicity to aquatic organisms. Alkalinity was shown to decrease acute Cu toxicity to fish and crustaceans (Miller and Mackay 1980; Gensemer, Naddy et al. 2002; Hyne, Pablo et al. 2005). This is likely related to complexation of Cu by higher concentrations of hydroxide and/or carbonate (USEPA 2007), which affects Cu bioavailability (Hyne, Pablo et al. 2005).

However, the protective effect of alkalinity may be confounded with the protective effect of Na, since these two parameters are commonly correlated. Increasing Na concentration decreases Cu toxicity to aquatic organisms, namely fish and crustacean species (Erickson, Benoit et al. 1996; De Schamphelaere and Janssen 2002a; Gensemer, Naddy et al. 2002; De Schamphelaere, Bossuyt et al. 2007; Ryan, Tomasso et al. 2009). The protective effect of Na on acute Cu toxicity seems to be observed ubiquitously (De Schamphelaere, Bossuyt et al. 2007) but is suggested to have an upper limit for some species (De Schamphelaere, Bossuyt et al. 2007).

Since Na is a cation it was expectable that Na increased competition with Cu for binding sites on the apical Na^+ channel occurs (Santore, Di Toro et al. 2001) what is also supported by other studies (e.g. De Schamphelaere and Janssen 2002a; De Schamphelaere and Janssen 2004c). However, at high Na concentrations, Cu uptake via apical Na^+ channel is barred, which points to the existence of other mechanisms for Cu uptake and toxicity. Following Grosell and Wood (2002), who demonstrated the presence of both a Na-sensitive and a Na-insensitive components in Cu uptake in fish (with the latter being increasingly important at elevated external Na concentrations), Borgmann and coworkers (2005) suggested that *D. magna* could also have two coexisting Cu uptake pathways, one Na-sensitive and the other one not Na-sensitive. This is supported by the fact that, being an essential element, Cu uptake must also occur for organisms acclimated to specific environmental conditions, such as high ambient Na concentration, where Cu uptake via Na^+

channel is blocked by Na (Komjarova and Blust 2009). This would allow the Cu uptake (although subjected to competition with Na^+) and further toxicity. However, other mechanisms were suggested to explain the protective effect of Na^+ on Cu toxicity (see De Schamphelaere, Bossuyt et al. 2007 for more details). For instance, additional Na is suggested to ameliorate the effect of Cu-induced Na loss across the gill via Na–K ATPase (De Schamphelaere and Janssen 2002a; Gensemer, Naddy et al. 2002), following the findings of previous studies with fish (Santore, Di Toro et al. 2001). Another probable mechanism is related to the reduced Cu uptake via the Na^+ channel, leading to a reduced inhibition of Na uptake rates (De Schamphelaere, Bossuyt et al. 2007).

Concerning potassium (K), another main cation usually present in natural waters, it was also tested as a competing ligand, but was found not to influence Cu toxicity to *Daphnia* (De Schamphelaere and Janssen 2002a).

Regarding anions not directly related to alkalinity, neither sulfate or chloride ions have been found to have any effect on acute copper toxicity to *Daphnia* in the ranges of ionic composition of natural waters (De Schamphelaere and Janssen 2004c).

As referred previously, Cu^{2+} is generally considered to be the most reactive and toxic Cu species. However, there is growing evidence that Cu toxicity is not determined by the free copper ion activity alone, but also by other Cu species. For instance, hydroxyl complexes ($CuOH^+$) and carbonate ($CuCO_3$) can also be bioavailable, particularly at high pH, but to a less extent than Cu^{2+} (De Schamphelaere, Heijerick et al. 2002). As a consequence, some authors suggested that total Cu concentration might be a better predictor of biological response (i.e. toxicity) than free Cu^{2+} activity (De Schamphelaere and Janssen 2002a).

1.4.3. Zinc toxicity

Most of the zinc (Zn) introduced into aquatic environments is adsorbed onto hydrous iron and manganese oxides, clay minerals, and organic materials and eventually is partitioned into the sediments (USEPA 1980). The distribution of Zn^{2+} over free ion (i.e., the hydrated ion $Zn(H_2O)_6^{2+}$) and complexed species in river water is found to be roughly 25-30% and 70-75%, respectively (Cleven and Van Leeuwen 1986; Jansen, van Leeuwen et al. 1998). Zinc can be adsorbed by organic ligands such as humic materials and biogenic structures

(i.e., cell walls of plankton) and by inorganic ligands such as mineral particles, clays, and hydrous oxides of manganese, iron, and silicon (Spear (1981), cited by Eisler 1993). Concerning the free ion concentration, usually considered the most toxic metal species, its proportion in typical freshwater conditions is much higher in the case of Zn rather than Cu: respectively 40% and 1% of the total metal concentration (Sigg and Xue 1994).

Acute Zn toxicity to freshwater fish is attributed to a competitively inhibited branchial Ca^{2+} influx that, in turn, leads to hypocalcaemia (Spry and Wood 1985; Hogstrand, Reid et al. 1995). The same mechanism of Zn toxicity is suggested to occur in *Daphnia* (Muyssen, De Schamphelaere et al. 2006). In fish, exposure to Zn can also cause disturbance of acid–base regulation and/or loss of sodium and chloride (Spry and Wood 1985). However, aquatic organisms usually have the ability to acclimate to sublethal concentrations of Zn (Alsop and Wood 2000; Muyssen and Janssen 2001).

Zn toxicity to freshwater aquatic organisms is modified by a number of water physicochemical parameters, including hardness (Pagenkopf 1983; Paulauskis and Winner 1988; Heijerick, De Schamphelaere et al. 2002; Heijerick, De Schamphelaere et al. 2005), dissolved organic matter (Paulauskis and Winner 1988; Oikari, Kukkonen et al. 1992; Bringolf, Morris et al. 2006) and pH (Heijerick, De Schamphelaere et al. 2002; Heijerick, De Schamphelaere et al. 2005; Hyne, Pablo et al. 2005). Figure 1.7 illustrates the BLM concept for Zn.

Increased hardness has a protective effect against Zn acute and chronic toxicity to aquatic organisms (Pagenkopf 1983; Paulauskis and Winner 1988; Barata, Baird et al. 1998; Heijerick, De Schamphelaere et al. 2002; Heijerick, De Schamphelaere et al. 2005). This protective effect is usually attributed to the competitive interaction between hardness cations and Zn for binding sites at the site of action (the Ca^{2+} channel) and also the effect of the water hardness on the general health of the daphnids (Heijerick, Janssen et al. 2003; Muyssen, De Schamphelaere et al. 2006). The protective effect of hardness is concordant with the decreased Zn uptake by *Daphnia* caused by increasing Ca concentration (Yu and Wang 2002; Komjarova and Blust 2009).

Concerning Ca and Mg, individually, their protecting effect on both acute and chronic Zn toxicity appears to be similar but of different magnitudes. For example, the effect of Mg was shown to be about 1/3 or 1/4 of the effect of Ca to *Daphnia* (Heijerick, De

Schamphelaere et al. 2005; Clifford and McGeer 2009) and fish (De Schamphelaere and Janssen 2004a), respectively.

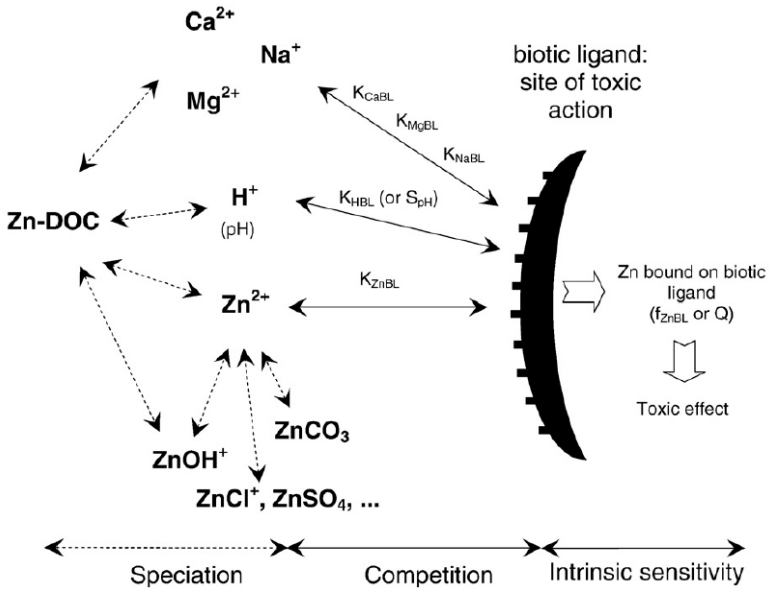


Figure 1.7: Schematic illustration of the biotic ligand model for Zn. Dashed arrows represent speciation of Zn, full lines represent competition with Zn for the biotic ligand (Van Sprang, Verdonck et al. 2009)

DOC or its humic fraction (humic acids), are both reported to decrease acute and chronic Zn toxicity, but the extent of the protective effect is somewhat controversial. Some studies report a major role of DOC on protecting from Zn toxicity (Heijerick, Janssen et al. 2003; Clifford and McGeer 2009), whereas others report a minor effect (Hyne, Pablo et al. 2005; Bringolf, Morris et al. 2006). The protective effect of DOC or humic acids is related to the complexation of Zn and, consequently, to the reduced bioavailability of the metal (Playle, Dixon et al. 1993b), the same effect that was referred for Cu in the previous section.

Concerning pH, its effects on Zn toxicity to crustaceans are contradictory. Some authors have shown that increasing pH reduces acute and chronic Zn toxicity (Heijerick, De Schamphelaere et al. 2002; Heijerick, Janssen et al. 2003; Clifford and McGeer 2009) due to a decreased predominance of the free metal ion (Schubauer-Berigan, Dierkes et al. 1993). Conversely, other studies have shown that increasing pH increases the toxicity of Zn

(Schubauer-Berigan, Dierkes et al. 1993; Heijerick, De Schamphelaere et al. 2005; Hyne, Pablo et al. 2005), which can be due to reduced metal uptake in consequence of decreasing competition between H^+ and Zn^{2+} for the receptor binding sites (Heijerick, De Schamphelaere et al. 2002; Paquin, Gorsuch et al. 2002).

The effect of Na was also found to be contradictory. Some studies report a protective effect of Na to acute and chronic toxicity to aquatic organisms (Heijerick, De Schamphelaere et al. 2002; De Schamphelaere and Janssen 2004a; Heijerick, De Schamphelaere et al. 2005), whereas in soft water the effect of Na was not significant on protecting *D. pulex* from Zn acute toxicity (Clifford and McGeer 2009).

Potassium, similarly to what was referred for Cu toxicity, does not affect Zn toxicity to *Daphnia* (Clifford and McGeer 2009).

1.5. Aims of the thesis

The main objective of the present thesis is to assess the ecological importance of the main water physicochemical parameters to life-history traits of crustaceans, either in the absence or presence of metals, using *Daphnia magna* as representative of aquatic crustaceans.

The effects of varying hardness, alkalinity, pH and temperature on growth, reproduction and intrinsic rate of population growth of *D. magna* were studied. Each of these water parameters was studied over a wide range of values in order to represent the variability in natural waters, not only geographical variability but also temporal variability related to phenomena such as acid deposition, hardness decline and global warming. In ecosystems, organisms are exposed to varying stressors that interact among each other, thus we focused on the study of varying two or more water parameters simultaneously.

In addition, the effects of water hardness and alkalinity on metals toxicity were also studied, using Cu and Zn as reference metals. Metals toxicity was assessed as acute effects (immobilization) and sublethal effects (feeding inhibition). Based on these data we developed simple mathematical models that can be used as preliminary tools in the assessment of the effects of metals under similar water chemistry conditions. This is particularly important for the effects of metals on feeding rate since, to the best of our knowledge, this topic was not studied previously.

1.6. Organization of the thesis

The present thesis is organized in six chapters. The first chapter provides an introduction to the theme of the present thesis, highlighting the relevance of studying varying water physicochemical parameters and their effects to aquatic crustaceans. Chapters 2 to 5 describe the results obtained during this study and are structured as scientific papers. Chapter 6 provides a general discussion of the results obtained.

Each chapter can be briefly summarized as follows:

- Chapter 1 provides a brief theoretical introduction regarding the variability and importance of water physicochemical parameters in aquatic ecosystems (with special relevance to hardness, alkalinity, pH and temperature) and metals toxicity, in particular Cu and Zn;
- Chapter 2 describes the combined effects of water hardness and alkalinity on growth, reproduction and population growth of *D. magna*;
- Chapter 3 describes the combined effects of water pH and hardness on growth, reproduction, feeding rate and population growth of *D. magna*;
- Chapter 4 addresses the combined effects of temperature and water chemistry parameters (hardness and alkalinity) on growth, reproduction and population growth of *D. magna*;
- Chapter 5 describes the effects of water hardness and alkalinity on the acute (immobilization) and sublethal toxicity (feeding inhibition) of Cu and Zn to *D. magna*, and presents the respective mathematical models;
- Chapter 6 provides a general discussion on the results obtained in Chapters 2 to 5. As each of these chapters includes its own discussion, here only a concise and integrative discussion of results is presented to highlight synergies among the different chapters and demonstrate the coherence of the work.

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CHAPTER

2

COMBINED EFFECTS OF VARYING WATER HARDNESS AND ALKALINITY ON LIFE-HISTORY TRAITS AND POPULATION GROWTH OF *DAPHNIA MAGNA*

2. Combined effects of varying water hardness and alkalinity on life-history traits and population growth of *Daphnia magna*

Abstract

In this paper we assessed the combined effects of varying water hardness and alkalinity on growth, reproduction and intrinsic rate of population growth of the aquatic crustacean *Daphnia magna*. An extensive range of hardness (0.5 - 3.6 mM $CaCO_3$) and alkalinity (0.3 - 2.3 mM $CaCO_3$) was tested, representing variability in natural waters. Hardness caused major effects on growth and development of *Daphnia*, whereas the effects of alkalinity were minor. Both hardness and alkalinity significantly affected reproduction, but the effect of hardness overruled the effect of alkalinity. In general, increasing hardness and alkalinity caused *Daphnia* to reproduce earlier and at a smaller size and increased the cumulative fertility. Moreover, water chemistry also affected the relationship body length - brood size. The intrinsic rate of population growth increased with alkalinity and was not affected by hardness. Moreover, data suggested an interaction between hardness and alkalinity on reproduction, suggesting that the effects of varying water hardness to *Daphnia* and probably other crustaceans might depend on the alkalinity level.

Keywords: hardness, alkalinity, *Daphnia magna*; growth; reproduction; population growth

2.1. Introduction

Water chemistry plays a crucial role in the diversity, abundance and health of organisms in aquatic systems. Water chemistry varies both spatially and temporally and is partly determined by environmental phenomena, such as acid deposition. Acid deposition leads to a reduction in the water pH and alkalinity. Additionally, it may also contribute to changes in water hardness by leaching Ca and Mg out of the adjacent soils (Watmough, Aherne et al. 2005; Cairns and Yan 2009). Initially, acid deposition increases soil base cations leaching and, consequently, their concentrations in aquatic systems. However, decades of acid deposition result in depletion of base cations in watershed soils and, thus, decreased

inputs to aquatic systems (Ashforth and Yan 2008). As a consequence, reduced hardness has been reported in many locations over Europe and North America over the past decades in surface waters (Skjelkvåle, Stoddard et al. 2005). However, to the best of our knowledge, the combined effects of varying water hardness and alkalinity on aquatic organisms have not been studied.

Crustaceans are ideal organisms to study the effects of water chemistry. They are vulnerable to the water hardness since Ca is a major component of their exoskeleton, which is shed regularly. *Daphnia* are considered the most vulnerable genus to low Ca concentration (Ashforth and Yan 2008). Moreover, crustaceans, including *Daphnia*, are widely distributed, and are amongst the most abundant zooplankton in many lake systems (Jeziorski, Yan et al. 2008). Additionally, they play a key role in the aquatic food webs: they are both principal prey for small fish and invertebrate predators, as well as the dominant algal grazers in many aquatic ecosystems. Thus, if major changes occur within crustacean communities there is potential of wide impacts in aquatic food webs (Jeziorski and Yan 2006). In this work we selected the species *Daphnia magna* as representative of freshwater crustaceans. This species is mainly found in eutrophic ponds, rock pools (Hebert 1978) and shallow lakes (Lampert 1991). These small-scale systems are particularly exposed to varying water chemistry since chemical variations are not buffered by the presence of a large water volume.

The effects of varying water chemistry on growth and reproduction of *Daphnia* have been addressed in previous studies. Decreasing hardness, mediated by decreasing Ca concentration, causes reduced body length, delayed sexual maturity and, thus, reduced reproduction (Cowgill and Milazzo 1991; Hessen, Alstad et al. 2000). Concerning alkalinity, Cowgill and Milazzo (1991a) observed that decreasing values caused no detrimental effects on the reproduction of *Daphnia* but an increase caused a slight delay in sexual maturity and a decline in reproduction. However, the effects of alkalinity on growth remain poorly understood. Concerning interaction effects among water chemistry parameters, Hooper and coworkers (2008) found a significant interaction between Ca concentration and pH on intrinsic rate of population growth of *Daphnia*. Since pH is correlated to alkalinity, these results might suggest that simultaneous variations of water

hardness and alkalinity might also have significant effects for *Daphnia* at the population level.

In this paper, we intend to answer the following question: Do simultaneous variations of water hardness and alkalinity have significant effects on growth and reproduction life-history traits of *Daphnia* that are relevant at the population level? To answer this question we performed life table experiments with *Daphnia magna* exposed to 11 different media, representing a wide range of natural waters. We focused on hardness between 0.5 and 3.6 mM as $CaCO_3$ and alkalinity between 0.3 and 2.3 mM as $CaCO_3$. The lowest Ca concentration used was 0.18mM, thus above the lower threshold suggested for *D. magna* survival and growth: 0.013 to 0.025 mM (Alstad, Skardal et al. 1999; Hessen, Alstad et al. 2000). The effects of varying water hardness and alkalinity were assessed on growth and development, reproduction life-history traits and intrinsic rate of population growth of *Daphnia*.

2.2. Material and Methods

2.2.1. Experimental organisms

Parent organisms originated from a single clone of *Daphnia magna* Straus (1820) clone F (sensu Baird, Barber et al. 1990). Organisms were individually cultured in 100 ml of ASTM hard water (ASTM 2004) with a standard organic additive (Marinure seaweed extract, Glenside Organics Ltd., UK) and fed 5.5 µg dry weight (dw)/ml/day of *Chlorella vulgaris* which were cultured in MBL medium. Culture medium was renewed every other day. Temperature was 20 ± 1ºC and photoperiod was 16 h light: 8 h dark.

Egg carrying females, 7-8 days old, were randomly assigned to each test medium and were cultured under the conditions described above. The first two batches of neonates (corresponding to the first two broods) were discarded. Neonates from third or fourth broods were used in the experiments as they were exposed to the test media during their entire developmental period (Barata, Baird et al. 2007).

2.2.2. Experimental design

Eleven synthetic media, with varying hardness and alkalinity, were selected from USEPA protocols for preparation of various synthetic freshwaters (USEPA 2002). Four hardness levels were considered: low (L), moderate (M), high (H) and very high (VH), corresponding to 0.5, 0.9, 1.7 and 3.5 mM as $CaCO_3$, respectively. Alkalinity levels were low (L), moderate (M), high (H) and very high (VH), corresponding to 0.3, 0.6, 1.1 and 2.3 mM as $CaCO_3$, respectively. Media are coded as "level of hardness. level of alkalinity". For example, the medium H.M refers to a medium with high hardness (1.7 mM) and moderate alkalinity (0.6 mM). Although hardness and alkalinity are expressed as concentration of $CaCO_3$, for simplicity we will omit "as $CaCO_3$" hereafter.

Within the possible ranges of hardness and alkalinity, not all combinations between hardness and alkalinity were tested because in natural waters there is a significant degree of covariability between hardness and alkalinity (Okland and Okland 1986). Thus, the ratio hardness/alkalinity was kept between 0.8 and 2.9, except for medium H.L (ratio = 5.4) and medium VH.L (ratio = 11.3). We included these two media as representative of atypical conditions.

Hardness and alkalinity of the test media were manipulated through the addition of different volumes of stock solutions of $CaSO_4.2H_2O$, $MgSO_4.7H_2O$, $NaHCO_3$ and KCl (analytical-grade) to ultrapure water. No pH adjustment was made except for the control medium (ASTM hard water). The measured chemical parameters of the test media are summarized in Table 2.1.

Table 2.1: Chemical properties of the test media: mean (SD), n≥12. C: control medium (ASTM hard water)

Medium	Hardness (mM $CaCO_3$)	Alkalinity (mM $CaCO_3$)	Conductivity ($\mu S\ cm^{-1}$)	pH	Concentration of major ions (mM)					
					Ca	Mg	Na	K	Cl	SO4
L.L	0.46 (0.02)	0.32 (0.02)	158.3 (16.9)	7.85 (0.21)	0.18 (0.01)	0.26 (0.02)	0.67 (0.06)	0.11 (0.01)	0.08 (0.02)	0.60 (0.04)
M.L	0.89 (0.03)	0.31 (0.02)	259.4 (5.0)	7.88 (0.17)	0.36 (0.01)	0.53 (0.02)	0.69 (0.02)	0.13 (0.01)	0.11 (0.01)	1.05 (0.04)
H.L	1.75 (0.06)	0.33 (0.02)	402.3 (39.7)	7.92 (0.21)	0.79 (0.08)	1.03 (0.12)	0.69 (0.08)	0.20 (0.02)	0.16 (0.02)	2.07 (0.10)
VH.L	3.56 (0.24)	0.32 (0.02)	631.4 (18.0)	7.76 (0.26)	1.34 (0.01)	2.48 (0.05)	0.74 (0.05)	0.30 (0.00)	0.22 (0.02)	3.99 (0.06)
M.M	0.90 (0.04)	0.58 (0.03)	291.6 (31.0)	8.08 (0.18)	0.37 (0.03)	0.51 (0.05)	1.25 (0.12)	0.13 (0.01)	0.10 (0.02)	1.10 (0.07)
H.M	1.75 (0.06)	0.62 (0.02)	468.7 (16.4)	8.22 (0.14)	0.77 (0.05)	1.07 (0.06)	1.32 (0.05)	0.20 (0.01)	0.16 (0.02)	2.03 (0.07)
M.H	0.89 (0.04)	1.12 (0.06)	389.4 (41.2)	8.34 (0.14)	0.37 (0.03)	0.51 (0.05)	2.41 (0.25)	0.19 (0.02)	0.16 (0.02)	1.12 (0.08)
C	1.74 (0.06)	1.06 (0.06)	555.9 (34.7)	8.10 (0.33)	0.75 (0.04)	1.05 (0.07)	2.52 (0.10)	0.19 (0.01)	0.30 (0.03)	2.04 (0.08)
H.H	1.75 (0.06)	1.14 (0.06)	546.4 (50.5)	8.34 (0.14)	0.76 (0.05)	1.00 (0.11)	2.44 (0.23)	0.20 (0.02)	0.16 (0.02)	2.08 (0.11)
H.VH	1.74 (0.06)	2.28 (0.08)	760.7 (25.3)	8.57 (0.14)	0.77 (0.06)	1.08 (0.06)	5.06 (0.20)	0.31 (0.02)	0.28 (0.03)	2.04 (0.11)
VH.VH	3.45 (0.10)	2.27 (0.12)	982.8 (92.6)	8.43 (0.16)	1.66 (0.22)	1.98 (0.24)	4.71 (0.52)	0.31 (0.03)	0.25 (0.04)	4.09 (0.22)

The experimental protocol followed OECD guideline 211 (OECD 2008). Tests were initiated with neonates (less than 24h) originated from parental daphnids acclimated to each of the experimental conditions tested, using twenty replicates per medium. Neonates were individually reared in glass vials containing 100 ml of test medium during 21 days. The algal and seaweed extract concentrations, as well as the photoperiod and temperature conditions were as described for culturing. Test media were renewed daily. During the media renewal, offspring as well as aborted eggs and embryos were counted and the shedded carapaces were collected for posterior determination of daphnids body length (BL). BL (from head to the base of spine) was estimated based on the length of the first exopodite of the second antennae (AL) which was measured in the carapace released at the end of each instar. The following equation was used: $BL = 10.98 \times AL - 0.55$ (r^2=0.978, n=128, p<0.0001). AL was measured under a stereomicroscope (MS5, Leica Microsystems, Houston, TX, USA) with a built-in calibrated eyepiece micrometer.

To illustrate the effects on somatic growth, the following endpoints are shown: body length (BL) at day 7, BL at day 14 and BL at day 21. The effects on development were assessed based on the number of juvenile instars (number of instars before eggs are first observed in the brood chamber).

The effects on reproduction were assessed by evaluating the following endpoints: age at first reproduction (AFR, age of the daphnid when the first brood is released); size at first reproduction (SFR, length of the daphnid when carrying the first brood in the brood chamber); fertility at first reproduction (FFR, number of viable juveniles produced in the first reproduction) and cumulative fertility (number of viable juveniles released during the 21 days period). We also assessed whether varying hardness and alkalinity affected the standard relationship between body length and brood size (Nogueira, Baird et al. 2004).

2.2.3. Chemical analyses

All chemical measurements were performed in both fresh and 24h-old media, i.e. before and after media renewal. Conductivity and pH were measured daily using a WTW Cond 330i meter, and a WTW pH 330 meter, respectively.

The concentration of major ions was determined in filtered samples (0.45µm cellulose acetate membrane). Chloride and sulfates were analyzed in Hach DR2000

spectrophotometer (Germany) using the mercuric thyocianate and the Sulfaver 4 methods, respectively. Cations (Ca, Mg, Na and K) were quantified in acidified samples using inductively coupled plasma mass spectrometry (ICP-MS Thermo Scientific X-Series) following ISO 17294.

Total hardness and total alkalinity were determined by the EDTA and the bromocresol green titrimetric procedures, respectively (American Public Health Association 2005).

2.2.4. Data treatment and statistical analyses

To assess the effects of water chemistry at the population level, the intrinsic rate of population growth (*r*) was determined using the Euler-Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x \qquad \text{(equation 2.1)}$$

where *x* is the age in days (0...n), lx is the age specific survivorship and mx is the number of newborns on day x.

Data for all endpoints were tested for normality and homoscedasticity using the Ryan-Joiner and the Levene's tests, respectively. When these conditions were met or a transformation was able to normalize the data, a one-way analysis of variance (ANOVA) was performed using the General Linear Model module of the statistical package Minitab© 15 (Minitab Inc., USA) and post-hoc comparisons were performed with the Tukey's test. Otherwise, data were analyzed with the nonparametric Kruskal-Wallis test and the post-hoc Dunn's test (SigmaStat 3.5, SPSS Inc., USA).

To assess the effects of hardness and alkalinity on the relationship body length – brood size we performed analysis of covariance (ANCOVA) following Zar (1999).

All statistical analyses were based on 0.05 significance level.

2.3. Results

Varying a water chemistry parameter causes a variation in other related parameters (Table 2.1), in a similar manner to natural waters. Increase in hardness and/or alkalinity is correlated to increased conductivity.

Growth, development, reproduction and intrinsic rate of population growth of *Daphnia* were significantly affected by the chemical parameters of the media (Table 2.2; Figures 2.1 to 2.6). More details on these and other endpoints are presented as Supplementary information.

Table 2.2: Probability values for the statistical testing of effects of hardness and alkalinity on growth and development, reproduction, and intrinsic rate of population growth of *D. magna*. N.S.: not significant

	Endpoint	p-value	
		hardness	alkalinity
growth and development	BL at day 7	≤0.001	N.S.
	BL at day 14	≤0.001	N.S.
	BL at day 21	≤0.001	0.021
	number of juvenile instars	≤0.001	≤0.001
reproduction	AFR	≤0.001	≤0.001
	SFR	≤0.001	≤0.001
	FFR	≤0.001	≤0.001
	cumulative fertility	≤0.001	0.030
population	intrinsic rate of population growth, r	N.S.	≤0.001

Growth and development

The effects of water chemistry on growth and development of daphnids are depicted in Figures 2.1 and 2.2. Hardness was determinant for somatic growth of daphnids, whereas alkalinity had a negligible effect (Table 2.2). Body length at start of the test (BL at day 0) varied between 0.8 and 0.9 mm and did not significantly differ among media. At the age of 7 days daphnids' body length varied between 2.6 and 3.2 mm, with higher values obtained in daphnids reared in media with low to moderate hardness. During the period 7 d to 14 d the growth trend was the opposite: daphnids in media with higher hardness grew more than daphnids in media with lower hardness. This caused a dissipation of the differences in daphnids body length determined at days 14 and 21 (Figure 2.1). Indeed, at day 14 body

length varied between 3.5 and 3.7 mm, and at day 21 body length varied between 3.9 and 4.1 mm, smaller variations than the observed at day 7. Daphnids reared in medium VH.L were always smaller than daphnids reared in other media.

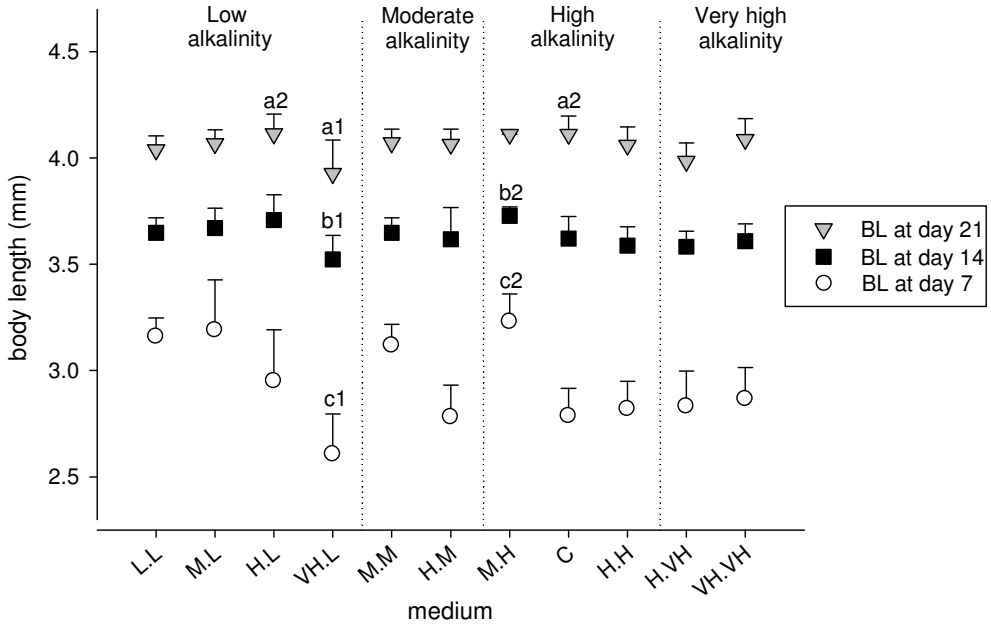

Figure 2.1: Body length of *D. magna* at day 7 (circles), day 14 (squares) and day 21 (triangles). Media are ordered by increasing alkalinity levels and, within each alkalinity level, by increasing hardness. Vertical bars represent 95% confidence interval of the mean. Symbols with different labels are statistically different among each other within a given alkalinity group. Only statistically conclusive comparisons are shown.

The major increment in length was registered in the first week (hatching to day 7), and decreased in posterior periods, evidencing that somatic growth rates decreases with increasing age in *Daphnia*.

The number of juvenile instars varied between 4.15 and 5.0 (Table 2.2). Both hardness and alkalinity affected this endpoint: decreasing hardness and alkalinity caused an increase in the number of juvenile instars (Figure 2.2).

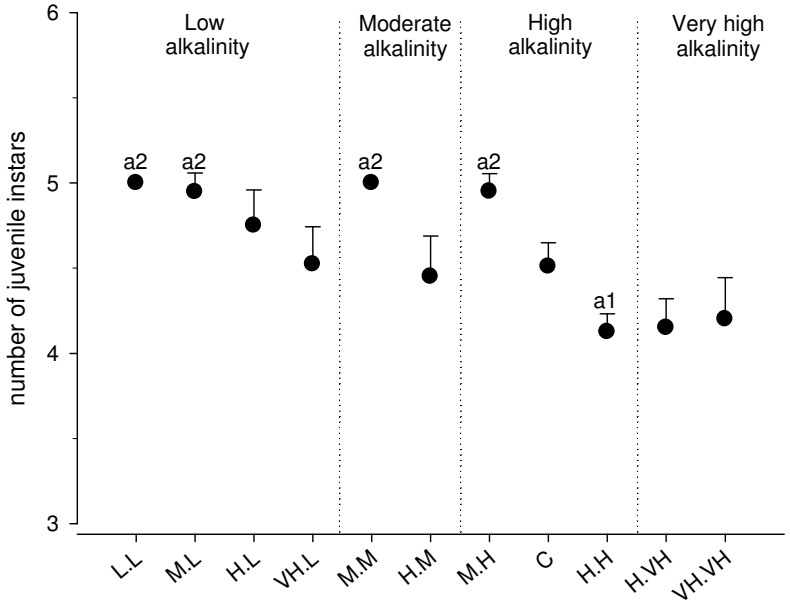

Figure 2.2: Number of juvenile instars of *D. magna* reared in different media. Media are ordered by increasing alkalinity levels and, within each alkalinity level, by increasing hardness. Vertical bars represent 95% confidence interval of the mean. Symbols with different labels are statistically different among each other. Only statistically conclusive comparisons are shown.

Reproduction

Age at first reproduction (AFR) and size at first reproduction (SFR) were affected by both hardness and alkalinity (Table 2.2), evidenced by the variation of these endpoints among media, which can be up to 1.5 days and 0.63 mm respectively for AFR and SFR (Figure 2.3). In general, increasing hardness and alkalinity leads daphnids to start reproducing earlier and at a smaller size. The reduced AFR with increasing hardness and alkalinity is concordant with the reduced number of juvenile instars. The effect of water chemistry is especially evident for AFR as regression analysis showed that hardness and alkalinity explain 43.6% of the variability in AFR: $AFR = -0.393 \times Hardness - 0.406 \times Alkalinity + 10.4$; adjusted-$r^2$ = 0.436; n=273; p<0.001).

SFR is dependent on AFR (the size of *Daphnia* depends on their age), which is corroborated by the similarity in the variation patterns of both endpoints (Figure 2.3). Although the effect of hardness was more evident in both AFR and SFR, it seems to be dependent of the alkalinity level. Figure 2.3 shows that the decrease in AFR and SFR

between media with high and very high hardness is more pronounced at low than at very high alkalinity levels.

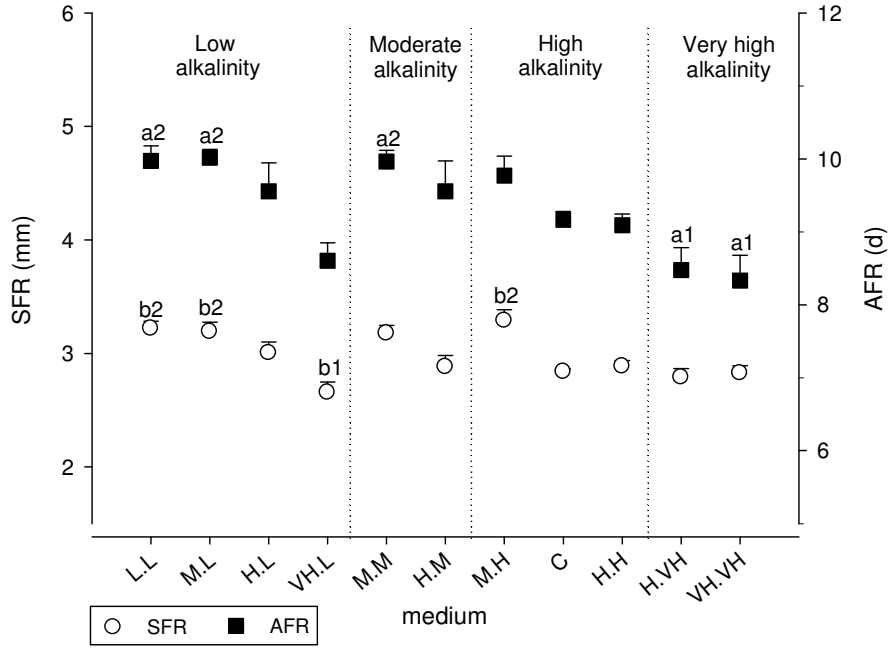

Figure 2.3: Size at first reproduction (SFR) and age at first reproduction (AFR) of *D. magna* reared in different media. Media are ordered by increasing alkalinity levels and, within each alkalinity level, by increasing hardness. Vertical bars represent 95% confidence interval of the mean. Symbols with different labels are statistically different among each other. Only statistically conclusive comparisons are shown.

Fertility at first reproduction (FFR) and cumulative fertility (Figure 2.4) were significantly affected by both hardness and alkalinity. FFR was higher in the daphnids reared in media with low to moderate hardness, which is greatly due to the increased SFR ($p \leq 0.001$), as supported by the similarity of the variation pattern between SFR and FFR. Hence, FFR can be predicted as a function of SFR ($FFR = 17.0 \times SFR - 34.7$; adjusted-$r^2$=0.641; n=195; $p<0.001$).

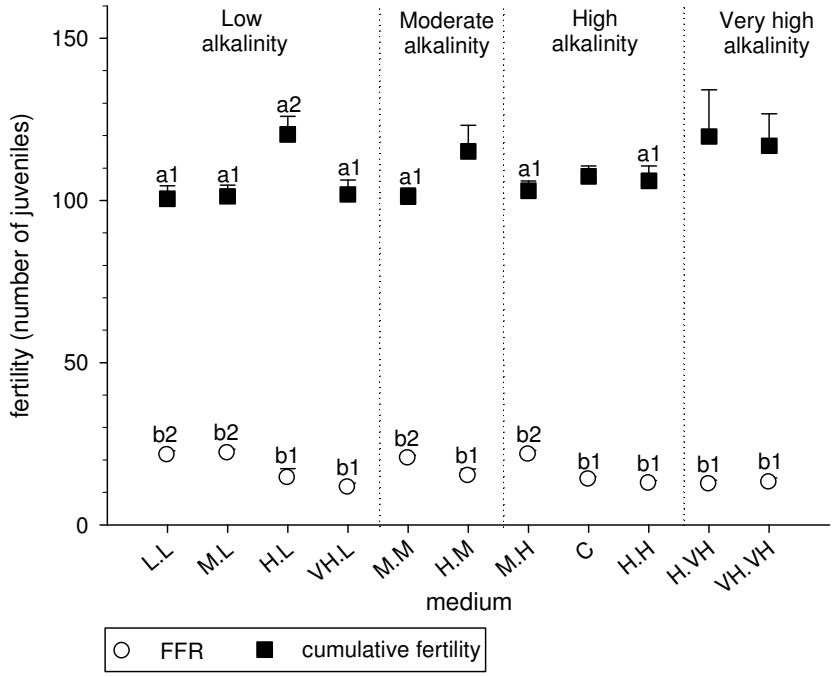

Figure 2.4: Fertility at first reproduction (FFR) and cumulative fertility of *D. magna* reared in each medium. Media are ordered by increasing alkalinity levels and, within each alkalinity level, by increasing hardness. Vertical bars represent 95% confidence interval of the mean. Symbols with different labels are statistically different among each other. Only statistically conclusive comparisons are shown.

Cumulative fertility, on the other hand, shows a variation pattern opposite to FFR: there is a slight trend for cumulative fertility to decline as hardness and alkalinity decrease, with the exception of medium VH.L. Indeed, despite the fact that daphnids reared in this medium started reproducing earlier and at a smaller size (Figure 2.3) they registered lower cumulative fertility values (Figure 2.4) and a lower mean brood size (Supplementary material). The variability in cumulative fertility is related to the number of broods: increasing hardness and alkalinity causes an increase in the number of broods (Supplementary material) and, thus, an increase of cumulative fertility.

The relationship body length - brood size was linear within the data range (Figure 2.5). Analysis of covariance showed that regression lines for the different test media are not coincidental ($F_{20;829}$=8.499; p<0.001). Significant differences among slopes were found ($F_{10;829}$=2.508; p=0.006) and the Tukey´s test for multiple-comparisons found significant

differences between the slope of medium L.L (slope = 12.22) and medium H.L (slope = 19.55), which have low alkalinity and different hardness levels.

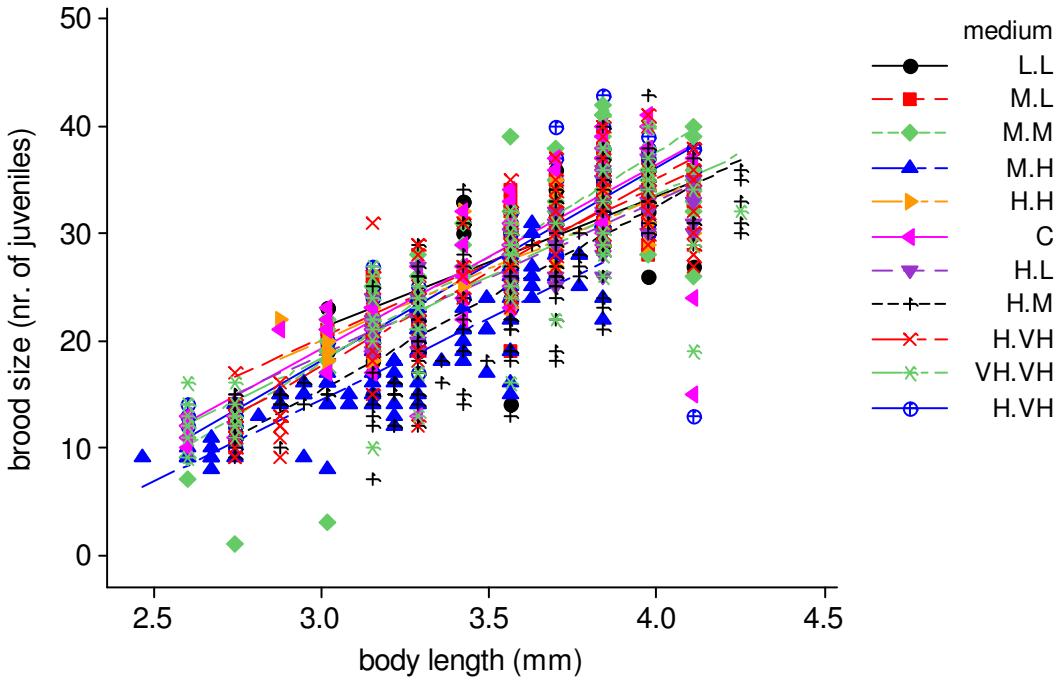

Figure 2.5: Relationship between body length and brood size of *D. magna* reared in different media.

Our data suggest an interaction effect between hardness and alkalinity. Concerning SFR, decreasing hardness from the very high level to the high level causes no significant differences at very high alkalinity, but a significant increase at low alkalinity (no differences between VH.VH and H.VH, but VH.L ≠ H.L). Furthermore, concerning cumulative fertility, decreasing hardness from the high level to the moderate level causes no significant differences at high alkalinity, but a statistically significant decrease at low alkalinity (no differences between H.H and M.H, but H.L ≠ M.L). These observations suggest that the effects of varying hardness are more pronounced at low alkalinity levels. On other hand, concerning AFR, decreasing alkalinity from the very high level to the low level causes no significant differences at very high hardness, but a significant increase at high hardness (no differences between VH.VH and VH.L, but H.VH ≠ H.L). This might suggest that the effects of varying alkalinity are dependent on the water hardness. However,

it was not possible to test the interaction effects between hardness and alkalinity since we did not carry out a full factorial design.

Population growth

The intrinsic rate of population growth (r) increased at very high alkalinity and was not affected by hardness (Table 2.2; Figure 2.6).

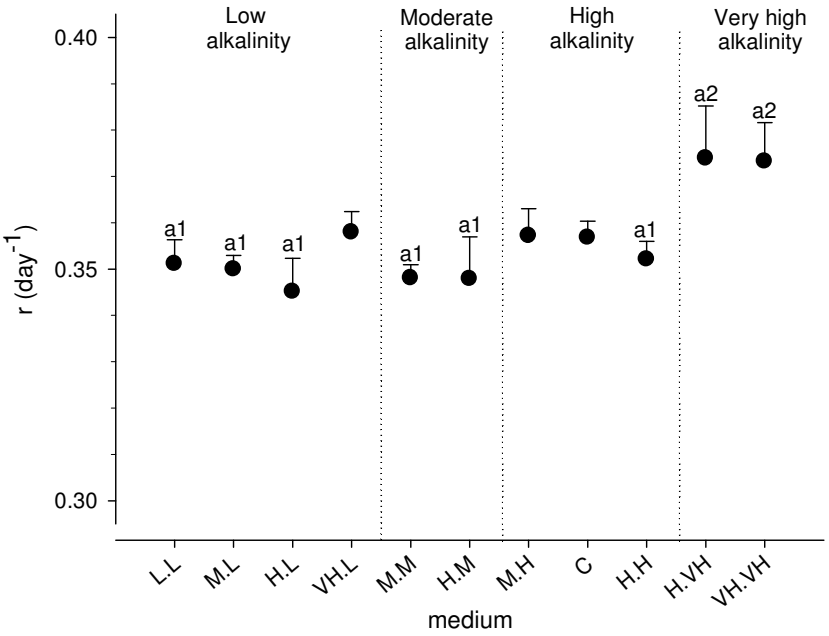

Figure 2.6: Intrinsic rate of population growth of *D. magna* reared in each medium. Vertical bars represent 95% confidence interval of the mean. Media are ordered by increasing alkalinity levels and, within each alkalinity level, by increasing hardness. Vertical bars represent 95% confidence interval of the mean. Symbols with different labels are statistically different among each other. Only statistically conclusive comparisons are shown.

2.4. Discussion

In this study we manipulated water hardness and alkalinity in order to study their combined effects on *Daphnia*. As in natural waters, varying a water chemistry parameter causes a variation in other related parameters. We emphasize that we intended to mimic natural

waters rather than studying the effect of varying a single water chemistry parameter keeping others constant. However, one has to be aware that, as in natural waters, the observed effects of hardness and alkalinity are related to the variation of other water chemistry parameters.

We carried out life table experiments with *D. magna* in order to study the effects of varying hardness and alkalinity on growth, development, reproduction and intrinsic rate of population growth of *Daphnia*. Our results showed that growth and development were mainly affected by hardness; reproduction was significantly affected by both hardness and alkalinity; and intrinsic rate of population growth was only affected by alkalinity.

Growth and development

The observed effects on growth agree with results of previous studies. An increase of 7d – old daphnids length with reduced hardness was also observed previously (Winner 1989; Muyssen, De Schamphelaere et al. 2009). This might seem contradictory to what was described in the Introduction. Indeed, a reduction in 7d-old daphnids body length is caused if Ca concentration decreases below the growth threshold (0.013 to 0.025 mM) (Hessen, Alstad et al. 2000), but above this threshold a reduction is caused by increasing Ca concentration (Winner 1989; Muyssen, De Schamphelaere et al. 2009). In aquatic systems the variability in daphnids body length after 7 days may result in competitive advantage over other zooplankton species, as will be discussed further. However, despite the variability in daphnids body length at day 7 (2.6 mm to 3.2 mm), these differences tended to dissipate (Muyssen, De Schamphelaere et al. 2009), and at day 21, daphnids body length varied only between 3.9 mm and 4.1 mm.

Daphnids in medium VH.L were smaller than the others throughout the test period. Medium VH.L has a high ratio hardness/alkalinity (11.3), corresponding to a ratio Na/Ca equal to 0.32 (in a molar basis). We believe that increased costs of maintenance in this medium might leave less energy available for growth, resulting in reduced body length. Additionally, the discrepancy in Na and Ca ionic concentrations might cause a reduced Na uptake. This explanation is in agreement with a previous study which reported a severe inhibition of Na uptake in the presence of high Ca conditions possibly mediated by

competitive interactions at a $2Na^+/H^+$ exchanger in daphnids' epithelia (Glover and Wood 2005).

In this study we showed that the number of juvenile instars in *Daphnia* was affected by water chemistry, decreasing with increasing hardness and alkalinity. The number of juvenile instars in *Daphnia* is known to be affected by temperature (Goss and Bunting 1983), BL at day 0 (Ebert 1994) and juvenile increment in body length which depends on food concentration (Ebert 1994). In this study the temperature and food concentration were constant, and BL at day 0 did not vary among test media; thus, the most likely explanation for the variability in the number of juvenile instars is that water chemistry affected the juvenile increment in body length (supported by Hessen, Alstad et al. 2000) and, consequently, the number of juvenile instars.

Reproduction

Reproduction was affected by both hardness and alkalinity, agreeing with findings from previous studies. AFR was reduced with increasing hardness (Cowgill and Milazzo 1991; Hessen, Alstad et al. 2000; Cairns and Yan 2009) and increasing alkalinity (Cowgill and Milazzo 1991a). Also, the variation in AFR was concordant with the variation in the number of juvenile instars.

The variation in AFR and SFR supports the trade-off between growth and reproduction (Enserink, Kerkhofs et al. 1995), commonly reported in *Daphnia*: daphnids that start reproducing earlier are smaller than daphnids that start reproducing later. The start of reproduction is a very important life-history trait in ecology (Enserink, Kerkhofs et al. 1995). In natural aquatic systems SFR plays a very important role in *Daphnia's* life-history due to size-selective predation (Lynch 1977). Daphnids that release the first brood at a smaller size are favored in aquatic systems where fish predation occurs; in opposition, fast-growing daphnids with a large SFR are favored in the presence of invertebrate predators that feed on small zooplankton (Lynch 1977). Thus, we may hypothesize that the water chemistry will potentially interfere with daphnids life-history traits under risk predation with consequences for population dynamics.

As expected, *D. magna* produced more progeny in hard water than in soft water (Cowgill and Milazzo 1991a). However, a further increase of hardness (from 1.7 mM to 3.5 mM)

caused a decrease in cumulative fertility at low alkalinity, which is concordant with the decrease in the number of offspring produced by *D. magna* in the first three broods when hardness exceeds 1.72 mM (Cowgill and Milazzo 1991a). However, since a similar increase in hardness at very high alkalinity caused no significant changes in cumulative fertility, our results suggest that the effects of varying hardness are more pronounced at lower alkalinity levels.

Concerning alkalinity, low levels are reported to have no effect on the cumulative fertility of *D. magna* (Cowgill and Milazzo 1991a), which agrees with our results, except for hardness level high (1.7 mM), which registered the highest cumulative fertility. This suggests an interaction effect between hardness and alkalinity. High alkalinity levels (equal or above 2.1 mM) are reported to reduce reproduction (Cowgill and Milazzo 1991a), which is not in agreement with our results, as we observed no reduction in the reproduction of daphnids reared at alkalinity 2.3 mM. This disagreement in results cannot be attributed to the hardness level, since both results refer to the hardness level 1.7 mM (high level, in this study). Eventually, differences may be related to the genetic variability in *D. magna* (Barata and Baird 1998) and/or to the experimental procedure. Previous studies show that daphnids acclimated to test conditions seem to exhibit less pronounced effects than non-acclimated daphnids (p.e. Leblanc and Surprenant 1984; Lasier, Winger et al. 2006). Note that in the present study an acclimation period to the test media was allowed, whereas Cowgill and Milazzo (1991a) allowed no acclimation. Thus, it is our belief that more pronounced differences among media would have been found if no acclimation period was allowed, due to immediate stress imposed to neonates at the start of the tests.

The enhancement of reproduction with increased hardness might be explained by an energy allocation that favors reproduction and maximizes daphnids' fitness (Nogueira, Baird et al. 2004) allied to increased feeding rate of daphnids (Muyssen, De Schamphelaere et al. 2009). This is translated to the increased slope of the relationship body length – brood size: at low alkalinity, high hardness favors the production of larger broods. The relationship body length – brood size is used to assess the reproductive potential of *Daphnia* populations (Hülsmann 2001). Both slope and intercept of this regression are affected by food quantity (Nogueira, Baird et al. 2004), food quality and possibly also by the size structure of the population (Hülsmann 2001). Moreover, this relationship is also affected by

water hardness, as shown in this study. This finding might be particularly relevant for the assessment of the reproductive potential of *Daphnia* populations.

Nevertheless, despite the very high hardness level, daphnids in medium VH.L registered the lowest cumulative fertility, which is, most probably, related to the reduced body length, as they were the smallest throughout the test period.

Our results suggest an interaction effect between hardness and alkalinity on reproduction which is in line with results of a previous study (Hooper, Connon et al. 2008). This is important since it gives evidence that the magnitude of the effects of hardness depend on the alkalinity level and vice-versa. Thus, in aquatic ecosystems, the effects of varying water hardness and alkalinity on reproduction of *Daphnia* and probably other crustaceans cannot be assessed independently of each other.

Population growth

The tested range of hardness had no significant effect on *r*, which agrees with the findings of Hooper and coworkers (2008) concerning Ca concentrations above 0.25 mM and pH 8. However, for hardness values outside the range we tested, *r*-values are expected to decrease (Cowgill and Milazzo 1991a; Hooper, Connon et al. 2008).

Increasing alkalinity significantly increased *r*, which is mainly derived from the earlier production of the first brood, demonstrating the importance of AFR for the population dynamics. Hooper and coworkers (2008) reported an increase in intrinsic rate of population growth with increasing pH from 7 to 8 but a further decrease when pH increased from 8 to 9, whereas we found an increase with increasing alkalinity, accompanied with an increase in pH from 7.8 to 8.6. Nonetheless, our results are not necessarily in contradiction with the results by these authors as a decrease in intrinsic rate of population growth might occur between pH 8.6 and 9.0. However, our results are in disagreement with the findings of Cowgill and Millazzo (1991a) who reported a steep decrease in *r* for alkalinity higher than 1.29 mM. The explanations for the possible divergences between our study and their study were referred previously and include clonal variability as well as the effect of acclimation.

The single effects of hardness and alkalinity to crustaceans species vary among species: although *Daphnia* are more sensitive to hardness than other crustaceans (Jeziorski, Yan et

al. 2008), they are less sensitive to alkalinity than other species (Cowgill and Milazzo 1991b). Thus, the effects on life-history traits of crustaceans may be different from the described here for *Daphnia*.

Besides the role of water hardness to *Daphnia* life-history traits, our results showed that alkalinity affects reproduction life-history traits of *D. magna*, particularly AFR and SFR, as well as the intrinsic rate of population growth. Also, our results suggest an interaction between water hardness and alkalinity. Thus, combined effects of varying hardness and alkalinity could have a significant impact on *Daphnia* population dynamics, which might have repercussions in aquatic food webs.

Acknowledgements

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Supplementary material

Table 2.3 presents the mean values of the different media concerning several endpoints, including some endpoints presented graphically in the paper.

Table 2.3: Mean values (SD in parenthesis) of several endpoints related to growth, reproduction and population growth of *D. magna* reared in several media.

	endpoint	medium										
		L.L	M.L	H.L	VH.L	M.M	H.M	M.H	C[1]	H.H	H.VH	VH.VH
growth and development	BL at day 7, mm	3.16 (0.03)	3.19 (0.08)	2.95 (0.06)	2.61 (0.04)	3.12 (0.03)	2.78 (0.03)	3.23 (0.04)	2.79 (0.02)	2.82 (0.02)	2.83 (0.04)	2.87 (0.03)
	BL at day 14, mm	3.65 (0.02)	3.67 (0.03)	3.71 (0.03)	3.52 (0.03)	3.65 (0.02)	3.62 (0.03)	3.73 (0.01)	3.62 (0.02)	3.59 (0.02)	3.58 (0.02)	3.61 (0.02)
	BL at day 21, mm	4.04 (0.02)	4.07 (0.02)	4.11 (0.02)	3.93 (0.04)	4.07 (0.02)	4.07 (0.02)	4.11 (0.00)	4.11 (0.02)	4.06 (0.02)	3.98 (0.04)	4.09 (0.03)
	number of juvenile instars	5.00 (0.00)	4.95 (0.05)	4.75 (0.10)	4.52 (0.11)	5.00 (0.00)	4.95 (0.05)	4.45 (0.11)	4.51 (0.07)	4.12 (0.05)	4.15 (0.08)	4.20 (0.12)
reproduction	age at maturity, d [2]	7.17 (0.05)	7.07 (0.05)	6.64 (0.15)	5.68 (0.12)	7.12 (0.00)	6.45 (0.19)	7.02 (0.10)	6.25 (0.05)	6.08 (0.07)	5.82 (0.07)	5.63 (0.14)
	AFR, d	9.97 (0.10)	10.02 (0.05)	9.56 (0.19)	8.60 (0.12)	9.96 (0.07)	9.56 (0.20)	9.77 (0.13)	9.16 (0.06)	9.09 (0.08)	8.48 (0.15)	8.33 (0.17)
	SFR, mm	3.22 (0.03)	3.19 (0.04)	3.00 (0.05)	2.66 (0.04)	3.18 (0.03)	2.88 (0.05)	3.29 (0.04)	2.84 (0.01)	2.89 (0.02)	2.79 (0.04)	2.82 (0.03)
	FFR	21.53 (0.62)	22.11 (0.59)	14.49 (1.34)	11.56 (0.66)	20.55 (0.43)	15.11 (1.02)	21.64 (0.64)	14.02 (0.41)	12.74 (0.47)	12.54 (0.58)	13.07 (0.65)
	cumulative fertility	100.52 (1.9)	101.27 (1.6)	120.39 (2.6)	101.84 (2.1)	101.22 (1.2)	115.12 (3.8)	103.00 (1.4)	107.39 (1.6)	106.00 (2.3)	119.67 (6.5)	116.87 (4.6)
	number of broods [3]	3.98 (0.00)	3.98 (0.00)	4.84 (0.09)	4.18 (0.00)	3.98 (0.00)	4.85 (0.09)	4.04 (0.05)	4.45 (0.06)	4.59 (0.09)	5.35 (0.19)	5.08 (0.14)
	mean brood size [4]	25.15 (0.47)	25.34 (0.41)	27.48 (0.52)	22.20 (0.46)	25.32 (0.31)	26.11 (0.53)	25.45 (0.27)	24.08 (0.28)	24.06 (0.35)	24.52 (0.69)	25.30 (0.52)
popu-lation	intrinsic rate of population growth, *r*	0.35 (0.00)	0.35 (0.00)	0.34 (0.00)	0.36 (0.00)	0.35 (0.00)	0.35 (0.00)	0.36 (0.00)	0.36 (0.00)	0.35 (0.00)	0.37 (0.00)	0.37 (0.00)

[1] control (ASTM hard water)

[2] Age at maturity: age of the daphnid when eggs are first observed in the brood chamber

[3] Number of broods: number of broods released by individual daphnids during 21 d

[4] Mean brood size: mean size of the broods released by individual daphnids

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CHAPTER

3

COMBINED EFFECTS OF WATER PH AND HARDNESS ON GROWTH, REPRODUCTION, FEEDING AND POPULATION GROWTH OF *DAPHNIA*

3. Combined effects of water pH and hardness on growth, reproduction, feeding and population growth of *Daphnia*

Abstract

All aquatic systems are subjected to changes in water chemistry parameters mainly due to environmental phenomena, such as acid deposition. Acid deposition leads to a simultaneous variation of water pH and hardness. Although the effects of pH and hardness on the life-history traits of *Daphnia* are known, little information is available regarding the combined effects of both water chemistry parameters. This work aims to fulfill this gap, providing a detailed description of the combined effects of varying water pH (5.7 to 9.0) and hardness (0.5 to 3.7 mM as $CaCO_3$) on the survival, growth, reproduction, feeding rate and population growth of the aquatic crustacean *Daphnia magna*. The effects of pH overruled the effects of hardness; however there seems to be an interaction between these parameters. Decreasing pH negatively affected the studied endpoints, but its effects were particularly harmful at low hardness, namely concerning survival, since daphnids reared at pH 5.7 and hardness 0.57 mM could not survive. This raises a concern in natural water bodies since soft water lakes are those at higher potential risk from acid deposition and declining Ca concentrations. In opposition, increasing pH to values about 9.0 may be harmful to *Daphnia*, in particular at very high hardness levels. Thus, in aquatic ecosystems the effects of varying water pH on life-history traits of *Daphnia* and other acid-sensitive crustaceans cannot be assessed independently of the hardness level. Decreasing pH in soft water lakes might be a major stressor to acid-sensitive crustaceans threatening their survival and, thus, the structure of aquatic food webs.

Keywords: pH; hardness; *Daphnia magna*; growth; reproduction; feeding rate; population growth

3.1. Introduction

All aquatic systems are exposed to changes in the water chemistry parameters, mostly as a consequence of environmental phenomena. One of the environmental phenomena which has affected many parts of Europe and North America in the last decades is acid deposition (Skjelkvåle, Stoddard et al. 2005). Acid deposition leads both to surface freshwater acidification and to variation of water concentration of calcium (Ca) and magnesium (Mg) (Skjelkvåle, Stoddard et al. 2005) i.e., variation of hardness. The variation of water hardness is related to leaching of base cations from watershed soils (Ashforth and Yan 2008). Initially, this causes an increase in water ion concentrations (increased hardness) but after decades of acid deposition this may result in the depletion of ions in watershed soils and hence decreasing hardness on aquatic systems (Ashforth and Yan 2008). Additionally, the decrease of ions in soils can be exacerbated by Ca loss due to forest biomass harvesting and regrowth after multiple timber harvesting cycles (Huntington, Hooper et al. 2000). Most water bodies in Europe and America are now "recovering" from acid deposition, as revealed by the tendency for increasing pH; however they are still facing a decrease in the concentration of Ca and Mg (Skjelkvåle, Stoddard et al. 2005). Declines in Ca concentration are of particular concern because of its implications for aquatic biota. First, many organisms, namely crustaceans, have relatively high Ca requirements and their abundance and distribution is dependent on Ca concentration (Jeziorski, Yan et al. 2008). Second, low Ca increases the susceptibility of aquatic biota to other stressors, such as acidity (Hooper, Connon et al. 2008), temperature (Ashforth and Yan 2008), UV radiation (Hessen and Rukke 2000) as well as to toxic metals (e.g. de Schamphelaere and Janssen 2002b). In addition, crustaceans, particularly *Daphnia*, are also highly sensitive to low water pH, due to the disruption of sodium (Na) metabolism at low pH (Glover and Wood 2005). Thus, *Daphnia* are usually not found at pH values below 5.8 (Almer et al. (1974). Although the effects of pH and hardness on the life-history traits of *Daphnia* are known, little is known about the combined effects of varying pH and hardness. Glover and Wood (2005) found that pH, Ca concentration and their interaction affected Na influx: lowering pH reduced Na uptake when Ca concentration was high (1mM), but had an opposite effect when Ca was absent. Furthermore, Hooper and coworkers (2008) suggested that *Daphnia*

population persistence is only possible above 0.0125 mM Ca and between pH 5.75 and 9.0, although more Ca is needed at lower pH values.

This work aims to assess the combined effects of varying water pH (5.7 to 9.0) and hardness (0.5 to 3.7 mM as $CaCO_3$) on the survival, growth, reproduction and feeding rate of the aquatic crustacean *Daphnia magna* and their repercussions at the population level. These ranges of water pH and hardness were selected as representative of a wide range of natural waters.

3.2. Material and Methods

3.2.1. Test organisms

Stock cultures originated from a single clone of *Daphnia magna* Straus (1820) clone F (sensu Baird, Barber et al. 1990) and were individually cultured in 100 ml of ASTM hard water (ASTM 2004) with a standard organic additive (Marinure seaweed extract, Glenside Organics Ltd., UK) and fed with 5.5 µg dry weight (dw)/ml/day of *Chlorella vulgaris* which were cultured in MBL medium. The photoperiod was 16h:8h (light: dark) and the temperature 20±1ºC.

Parental daphnids were acclimated to the test conditions: pH and hardness. Egg carrying females, 7-8 days old, were randomly assigned to each medium and cultured under the photoperiod and temperature conditions described above. The first two broods were discarded. Only neonates from third or fourth broods were used in the experiments as these organisms were exposed to the test conditions during their entire developmental period, according to Barata and Baird (2000).

3.2.2. Experimental design

Media were prepared by adding different volumes of stock solutions of $NaHCO_3$, $MgSO_4.7H_2O$, KCl and $CaSO_4.2H_2O$ (analytical-grade, Merck, Germany) to ultrapure water. Three hardness levels were tested: low, high and very high, corresponding to 0.5, 1.9 and 3.7 mM as $CaCO_3$, respectively. Although hardness is expressed as a concentration of $CaCO_3$, for simplicity "as $CaCO_3$" will be omitted hereafter. Four nominal pH levels were selected: 5.7, 6.7, 7.7 and 9.0. In order to stabilize pH different buffers were used:

MES.H_2O (2-morpholinoethanesulfonic acid, Sigma-Aldrich, Germany, at a concentration of 2.22mM) was used to stabilize pH 5.7 and 6.7; $NaHCO_3$ was used for pH 9.0; no pH buffer was added to stabilize pH at 7.7. The pH adjustments were performed using HCl or NaOH. Chemical stabilization of the media was allowed overnight. New media were prepared every other day, stored at 20ºC and used to renew daphnids media. The measured chemical parameters of the test media are summarized in Table 3.1. Media were coded as follows: "level of hardness - nominal pH", e.g. the medium H-6.7 refers to a medium with high hardness (1.7 mM) and nominal pH 6.7. The control medium was ASTM hard water adjusted to pH 7.8 ± 0.1 (mean ± SD) with HCl (ASTM 2004), and corresponds to medium H-7.7.

3.2.3. Chronic exposure tests

The experimental protocol followed OECD guideline 211 (OECD 2008). Ten neonates (less than 24 h old, one daphnid/beaker) were transferred to glass beakers containing 100 ml of the selected medium. The temperature was 20±1ºC and the light:dark cycle was 16h:8h. Twice a day (12 h accuracy), during 21 d, daphnids were checked for mortality, reproduction and growth. Media were renewed every other day, including the addition of the standard organic additive which represented 3.1 mg DOC/L. The algae *C. vulgaris* was added daily at a concentration of 5.5 µg dw/ml). During media renewal all live offspring and aborted eggs and embryos in each vial were counted and the shedded carapaces collected. Body length was determined indirectly from the length of the first exopodite of the second antennae measured from daphnids' molted carapaces (as described in Chapter 2) in order to avoid stressing test organisms. The body length was determined in at least 5 individuals per instar per treatment. Additionally, body length of neonates (from top of the head to the base of the tail) from the third brood was measured, in order to determine the maternal investment in reproduction. Both the measurements of antennae from daphnids carapaces and the body length of the neonates were performed under a stereomicroscope (MS5, Leica Microsystems, Houston, TX, USA) with a built-in calibrated eyepiece micrometer.

Table 3.1: Chemical properties of the test media (mean values, n≥5).

medium	hardness (mM as $CaCO_3$)	alkalinity (mM as $CaCO_3$)	pH	conductivity (mS/cm)	major ions (mM)					
					Ca	Mg	Na	K	SO_4	Cl
L - 6.7	0.57	0.89	6.64	208.7	0.21	0.27	2.15	0.13	0.66	0.09
L -7.7	0.53	0.33	7.74	146.1	0.21	0.26	0.84	0.12	0.65	0.08
L - 9	0.52	0.92	8.41	233.4	0.20	0.28	2.06	0.11	0.67	0.08
H - 5.7	1.93	0.29	5.78	543.6	0.74	1.13	2.93	0.20	2.12	1.73
H - 6.7	1.90	1.06	6.72	490.2	0.79	1.22	3.17	0.23	2.12	0.47
H - 7.7	1.85	1.05	7.75	522.9	0.76	1.21	2.99	0.21	2.01	0.41
H - 9	1.77	3.76	8.95	840.6	0.70	1.00	8.18	0.19	2.17	0.16
VH - 5.7	3.74	0.26	5.73	627.3	1.58	2.61	0.96	0.36	3.99	0.34
VH - 6.7	3.70	0.87	6.64	690.5	1.62	2.53	2.42	0.35	4.03	0.24
VH - 7.7	3.75	0.33	7.67	639.8	1.61	2.47	0.90	0.36	3.99	0.24
VH - 9	2.93	5.67	8.96	1363.0	0.93	1.98	13.89	0.32	3.89	0.24

The combined effects of water pH and hardness on somatic growth were assessed by determining the initial body length (at start of the tests) and final body length (at day 21). The effects on reproduction were assessed by evaluating the following endpoints: age at first reproduction (AFR, age of the daphnid when the first brood is released); size at first reproduction (SFR, length of the daphnid when carrying the first brood in the brood chamber); fertility at first reproduction (FFR, number of viable juveniles produced in the first reproduction) and cumulative fertility (number of viable juveniles released during the 21 days period). The maternal investment in reproduction was determined as maternal investment per neonate (as average neonate size). The effects at the population level were assessed by determining the intrinsic rate of population growth.

3.2.4. Feeding inhibition tests

Feeding inhibition tests were performed with daphnids at two different life stages: juveniles (fourth instar) and adults (after releasing the first brood). In both cases, organisms originated from acclimated parental daphnids.

Tests were carried out in plastic vials containing 50 ml of the test medium and the algae *C. vulgaris* at 5.5 µg dw/ml. Each treatment consisted of five replicates and four blanks (having no daphnids). In the tests with juveniles 3 organisms per replicate were used, whereas in the tests with adults 2 organisms per replicate were used. The vials were kept 6 hours in the dark at 20 ±1ºC. These test conditions were shown to elicit a significant feeding of the daphnids, preventing both settlement of the algae and starvation of the daphnids (unpublished data). After the exposure period daphnids were removed, the vials were vigorously shaken and optical density was measured in a UV-spectrophotometer (Jenway 6505 Spectrophotometer ultraviolet-visible) at 440 nm. The body length of daphnids was estimated based on the length of the first exopodite of the second antennae, as described previously. Feeding rates were calculated using the equation by Allen and coworkers (Allen, Calow et al. 1995), with slight adaptations:

$$F = \frac{V(C_0 - C_t)}{tN} \qquad \text{(equation 3.1)}$$

where:

F = feeding rate of single organisms (µg dw/ind/h)

V = volume of medium (ml)

C_0 = final cell concentration in the blanks (µg dw/ml)

C_t = final cell concentration in the treatment (µg dw/ml)

t = time organisms were allowed to feed (h)

N = number of organisms per replicate

The combined effects of pH and hardness on feeding rates of juveniles or adults were assessed by determining the feeding rates relative to the control. Relative values were calculated dividing the absolute feeding rates by the average of the feeding rates of control daphnids. This allowed the direct comparison of values obtained in different experiments.

3.2.5. Chemical measurements

All chemical measurements were performed in fresh and 48h-old medium, i.e. before and after renewal of the test media. Conductivity, dissolved oxygen, pH and temperature were measured every other day, using a WTW Cond 330i meter, a WTW Oxi 330 meter and a WTW pH 330 meter, respectively.

The concentration of major ions was determined in filtered samples (0.45µm cellulose acetate membrane) collected randomly throughout the test period. Anions were quantified using a Hach DR2000 spectrophotometer (Germany): chloride was determined by the mercuric thyocianate method and sulfates by the Sulfaver 4 method. The cations calcium, magnesium, sodium and potassium were quantified in acidified samples using inductively coupled plasma mass spectrometry (ICP-MS Thermo Scientific X-Series).

Total hardness and total alkalinity were measured twice a week, employing the EDTA and the bromocresol green titrimetric procedures, respectively (American Public Health Association 2005).

Dissolved organic carbon was quantified with a Sievers 800 Portable TOC analyzer in filtered samples (0.45 µm polypropylene membrane) which were collected randomly throughout the test period.

3.2.6. Data treatment and statistical analyses

The intrinsic rate of population growth (*r*) was determined following the Euler-Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x \qquad \text{(equation 3.2)}$$

where *x* is the age in days (0...n), l_x is the age specific survivorship and m_x is the number of newborns on day x.

Although we planned a full factorial experimental design, such design was not possible because the parental daphnids exposed to medium L-5.7 (low hardness and pH 5.7) did not survive the acclimation period, as a consequence of the test conditions. Since we did not carry out a full factorial design we could not use 2-way ANOVA to test interaction effects of pH and hardness on the studied endpoints. Instead, we performed 1-way ANOVA using the General Linear Model module of the statistical package Minitab© 15 (Minitab Inc., USA) including in the model both pH and hardness, in order to test the significance of pH and hardness to each studied endpoint. Additionally, we performed 1-way ANOVA for each hardness level to test the effects of pH, and multiple comparisons were performed with the Tukey's test, using the SigmaPlot statistics package (SigmaStat 3.5, SPSS Inc., USA). Data for all endpoints were tested for normality and homoscedasticity using the Ryan-Joiner and the Levene's tests, respectively. Heteroscedastic variables or with a non-normal distribution were analyzed with the nonparametric Kruskal-Wallis test and the post-hoc Dunn's test (SigmaStat 3.5, SPSS Inc., USA). All statistical analyzes were based on 0.05 significance level.

3.3. Results

Increasing pH to the highest values caused a reduction in hardness and concentration of Ca and Mg, most evident at the very high hardness (Table 3.1). This is due to chemical

precipitation of Ca and Mg, probably as carbonates, which is corroborated by the formation of a fine white layer in the test vials.

The effects of pH and hardness on each endpoint are illustrated in Figures 3.1 to 3.7. Table 3.2 shows the results of the statistical analysis to the effects of pH and hardness on each endpoint.

Survival

The survival of daphnids was affected by pH. Parental daphnids reared at pH 5.7 and low hardness (about 0.5 mM $CaCO_3$) did not survive the acclimation period. Additionally, mortality at pH 5.7 occurred also for hardness levels 1.9mM (6 daphnids in 10) and 3.5mM (5 daphnids in 10).

Growth

The effects of pH and hardness on daphnids body length (BL) at day 0 and day 21 are shown in Figure 3.1. BL at day 0 was significantly affected by both pH and hardness (Table 3.2). BL at day 21 varied more widely than BL at day 0 and was affected by both pH and hardness, although the effect of pH was more evident: increasing pH caused an increase in BL at day 21. Our results also suggest an interaction between these parameters, since increasing hardness tended to dissipate the effects of varying pH on BL at day 21, i.e., increased the BL at day 21 at low pH and decreased it at high pH.

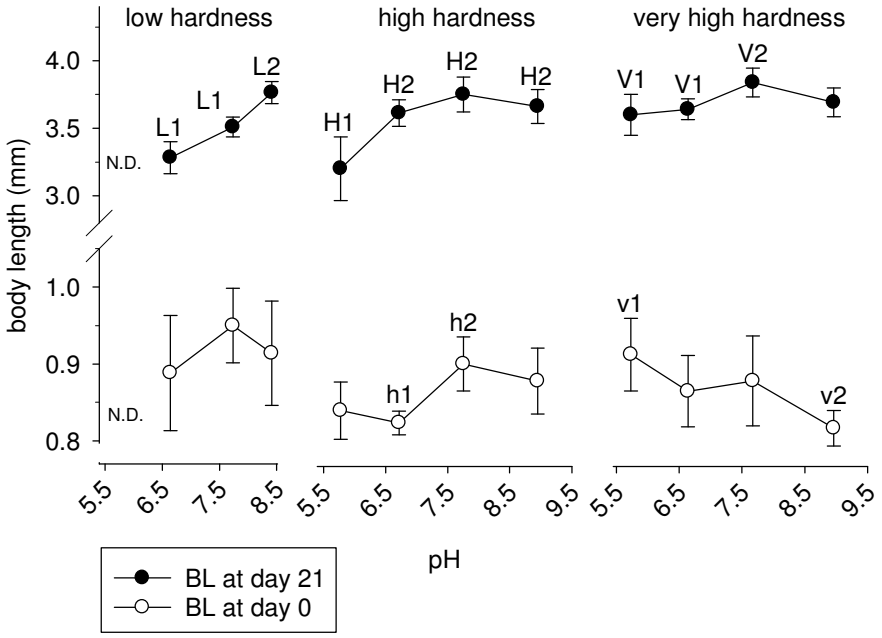

Figure 3.1: Body length (BL) of *D. magna* at the start of the test (BL at day 0) and after 21 days (BL at day 21) as a function of pH and hardness. Vertical bars represent 95% confidence interval of the mean. Within each hardness level, symbols with different labels are statistically different among each other. Only statistically conclusive comparisons are shown. N.D.: not determined due to mortality of parental daphnids

Reproduction

The effects of varying pH and hardness on age at first reproduction (AFR) and size at first reproduction (SFR) are depicted in Figure 3.2. Mean AFR varied between 9 and 11 days and did not follow a clear pattern. Significant differences due to pH were only found at low hardness, which suggests an interaction between pH and hardness. SFR, on the other hand, was clearly affected by pH: rising pH increased SFR at any hardness level. Additionally, increased hardness also increased SFR, although this effect was less evident than the pH effect. It is interesting to note that, although daphnids reared in medium L-6.7 had the highest AFR, they had one of the lowest SFR.

Table 3.2: Probability values for the statistical testing (one-way ANOVA) on the effects of pH (measured values) and hardness on several endpoints of *D. magna*. AFR: age at first reproduction; SFR size at first reproduction; FFR: fertility at first reproduction: N.S.: not significant

	endpoints	significant covariates	hardness	pH (hardness)
growth and development	BL at day 0	---	0.037	< 0.001
	BL at day 21	---	0.030	< 0.001
reproduction	AFR	---	< 0.001	< 0.001
	SFR	---	0.026	< 0.001
		age (p<0.001)	N.S.	< 0.001
	FFR	---	N.S.	< 0.001
		SFR (p<0.001)	N.S.	0.007
	cumulative fertility	---	0.016	< 0.001
		AFR (p<0.001)	N.S.	< 0.001
	average brood size	---	0.039	< 0.001
	3rd brood neonate size	---	< 0.001	< 0.001
		mother size (p=0.044)	< 0.001	< 0.001
feeding rate (FR)	relative FR of juveniles	---	< 0.001	< 0.001
	relative FR of adults	---	0.031	< 0.001
population	intrinsic rate of population growth, *r*	---	0.007	< 0.001

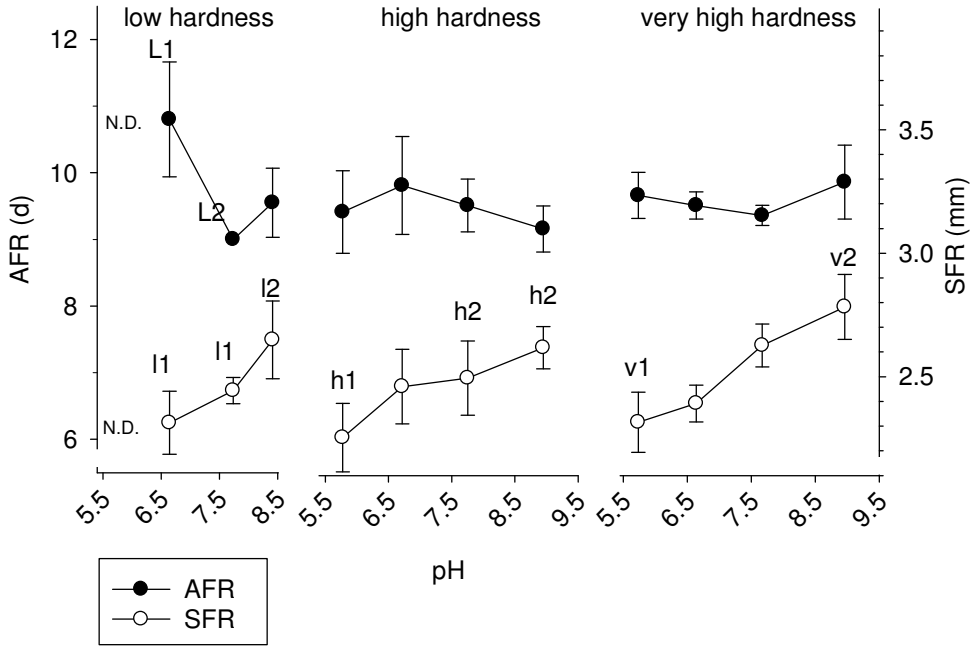

Figure 3.2: Age at first reproduction (AFR) and size at first reproduction (SFR) of *D. magna* as a function of pH and hardness. Vertical bars represent 95% confidence interval of the mean. Within each hardness level, symbols with different labels are statistically different among each other. Only statistically conclusive comparisons are shown. N.D.: not determined due to mortality of parental daphnids

The effects on fertility at first reproduction (FFR) and cumulative fertility of *D. magna* are depicted in Figure 3.3. FFR increased with increasing pH at low and high hardness but was not affected by pH at very high hardness. Water hardness *per se* did not affect FFR (Table 3.2). As expected, FFR was significantly affected by SFR (Table 3.2), which is concordant with the similar pattern observed for both endpoints particularly in function of pH.

Concerning cumulative fertility, mean values varied between approximately 30 and 70 juveniles. As FFR, cumulative fertility increased with increasing pH at low and high hardness but was not affected by pH at very high hardness. Considering the significant effects of AFR on cumulative fertility, hardness becomes non-significant, suggesting that the effects of hardness on cumulative fertility are indirect effects through AFR. For a certain pH, cumulative fertility tends to increase as hardness increases. However, at pH 9.0 this tendency is not observed, which suggests an interaction between these water chemistry parameters.

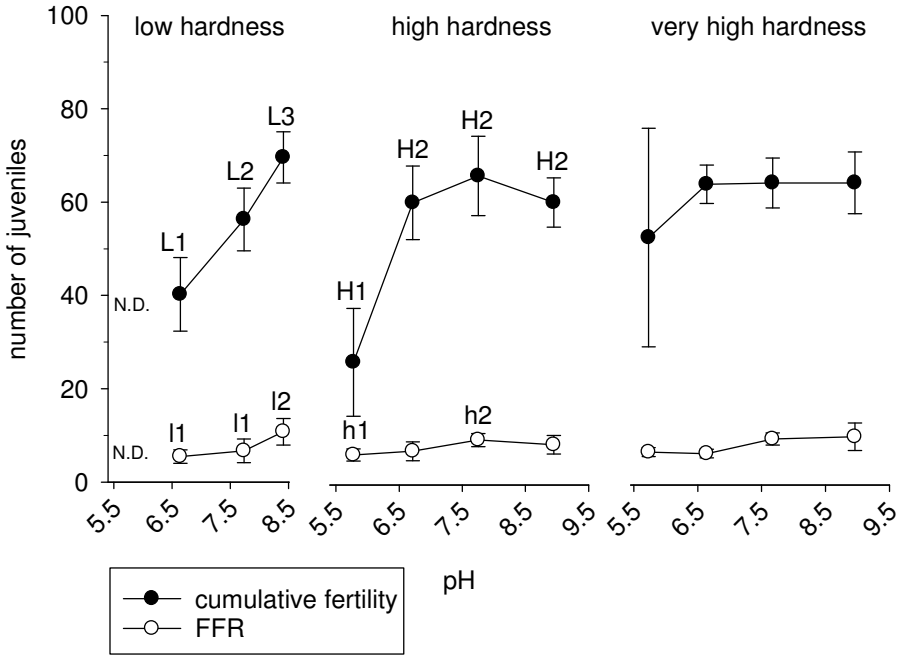

Figure 3.3: Cumulative fertility and fertility at first reproduction (FFR) of *D. magna* as a function of pH and hardness. Vertical bars represent 95% confidence interval of the mean. Within each hardness level, symbols with different labels are statistically different among each other. Only statistically conclusive comparisons are shown. N.D.: not determined due to mortality of parental daphnids

The maternal investment per neonate was also affected by the water chemistry parameters, as illustrated in Figure 3.4 and Table 3.2. In general, the average size of 3rd brood neonates increased with increasing pH, despite a small decrease at pH 7.7. Neonate size was affected by mother size, although to only a small extent. For instance, in the pH range between 6.7 and 7.7 neonate size tended to decrease, despite the increase of mother size. Additionally, neonate size varied inversely with the feeding rate of adults (except at very high hardness): feeding rate of adults are higher at pH 7.7, whereas neonate size are lower.

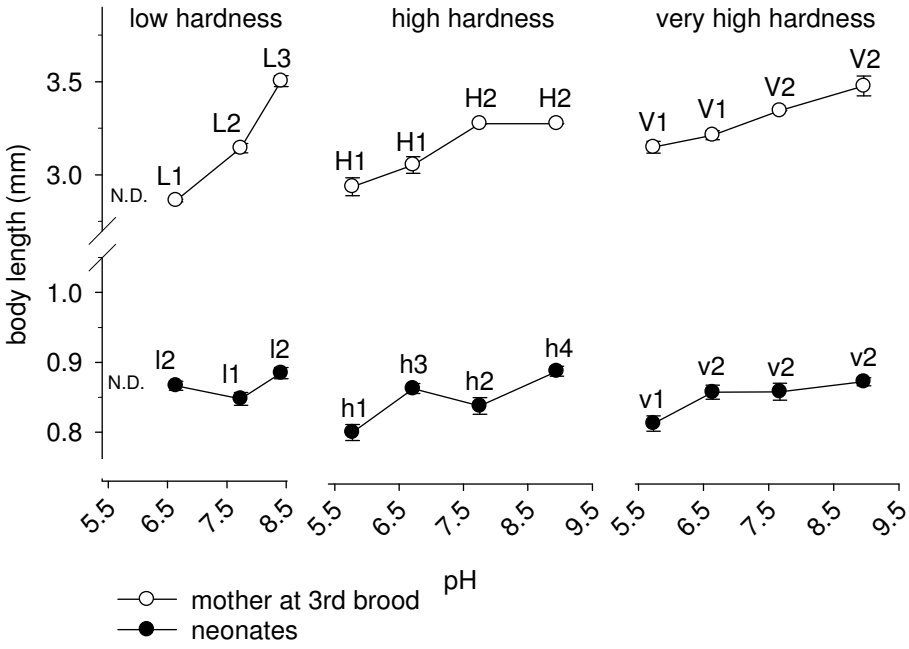

Figure 3.4: Size of 3rd brood neonates and respective mothers as a function of pH and hardness. Vertical bars represent 95% confidence interval of the mean. Within each hardness level, symbols with different labels are statistically different among each other. Only statistically conclusive comparisons are shown. N.D.: not determined due to mortality of parental daphnids

Feeding rate

The feeding rates of juvenile and adult daphnids relative to the control (Figure 3.5) were affected by pH and hardness, although the effect of hardness on the feeding rate of adults was diminutive (Table 3.1). The relative feeding rates of both juveniles and adults followed a very similar pattern: increased with pH but registering a maximum at pH 7.7; the exception was the relative feeding rate of juveniles at low hardness which increased until the highest pH and, thus did not have a maximum at pH 7.7.

Unexpectedly, the feeding rates of juvenile and adult daphnids were not significantly affected by the daphnids body length ($p>0.05$). For instance, despite the large size of daphnids reared at the highest pH, the feeding rates were frequently low.

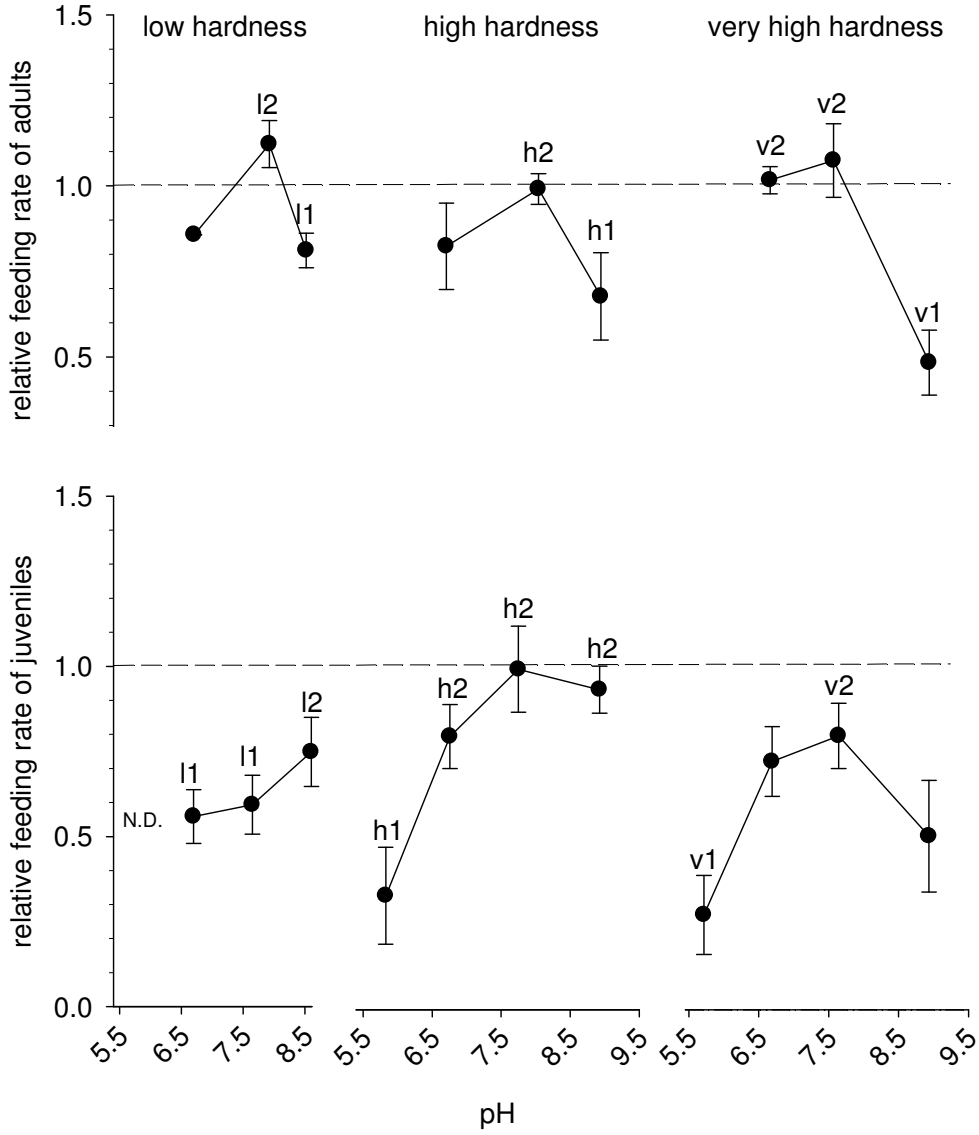

Figure 3.5: Relative feeding rates of adults and juveniles of *D. magna* as a function of pH and hardness. Vertical bars represent 95% confidence interval of the mean. Within each hardness level, symbols with different labels are statistically different among each other. Only statistically conclusive comparisons are shown. N.D.: not determined due to mortality of parental daphnids

Intrinsic rate of population growth

The intrinsic rate of population growth (r) of daphnids increased with increasing pH and hardness (Figure 3.6, Table 3.2). However, at very high hardness, increases in pH above 7.7 have a different behavior, which suggests an interaction effect between pH and hardness.

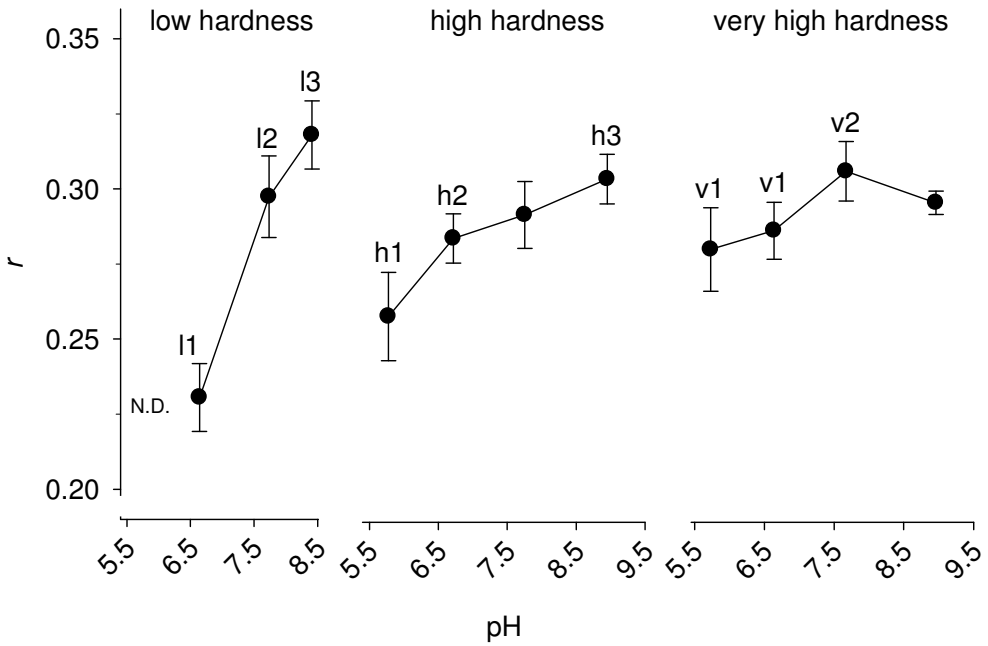

Figure 3.6: Intrinsic rate of population growth (*r*) of *D. magna* as a function of pH and hardness. Vertical bars represent 95% confidence interval of the mean. Within each hardness level, symbols with different labels are statistically different among each other. Only statistically conclusive comparisons are shown. N.D.: not determined due to mortality of parental daphnids

3.4. Discussion

In this study we observed that variations in pH between 5.7 and 9.0 were more determinant for *D. magna* than variations in hardness between 0.5 and 3.5 mM. The high sensitivity of *D. magna* to low pH is due to the effects of H^+ on their finely balanced sodium metabolism (Glover and Wood 2005). Sodium uptake is reduced as pH decreases, due to a mechanism of proton-linked sodium uptake (Glover and Wood 2005). Such mechanism explains the high sensitivity of daphnids (as well as other aquatic organisms) to aquatic acidification and, thus, the decline of *Daphnia* populations in acidified lakes (Locke and Sprules 2000; Jeziorski, Yan et al. 2008).

We showed that low pH is particularly harmful at low hardness, namely concerning survival, which represents a reason for concern in natural water bodies. Soft water lakes are at higher potential risk from acidification (Lonergan and Rasmussen 1996) and decreasing

Ca concentration (Jeziorski, Yan et al. 2008) and, as we showed, are also those in which the effects of decreasing pH are more adverse. Thus, decreasing pH in soft water lakes might be a major stressor not only to *Daphnia* but also to other acid-sensitive species threatening not only their survival but also, indirectly, other species by affecting the structure of aquatic food webs.

Increasing pH to values about 9.0 seems to cause adverse effects to *Daphnia*, in particular at very high hardness levels. This toxicity probably derives from the high Na concentrations and/or ionic imbalance. Indeed, at the highest pH, Na concentration was 10 to 15 times higher than Ca concentration in a molar basis (low and very high hardness, respectively), which might have caused a reduced sodium uptake (as reported by Glover and Wood 2005) and, consequently, increased costs of maintenance. It is important to mention that this scenario, although not favored by environmental phenomena, is likely to occur due to the correlation of pH and hardness in natural waters (Okland and Okland 1986), of which the south of Portugal and Spain are good examples.

The studied endpoints were negatively affected by decreasing pH, which is in line with the results of previous studies (Walton, Compton et al. 1982; Havas 1985). The decrease in cumulative fertility, for instance, was accompanied by increased AFR and reduced FFR (Walton, Compton et al. 1982), in particular at low hardness levels. One interesting detail is the high variability in cumulative fertility of daphnids reared in medium VH-5.7. This variability is due to the fact that, despite the lower cumulative fertility of some daphnids, other daphnids could produce as many juveniles as unstressed daphnids. This observation agrees with that of Walton and coworkers (1982) who reported that reproduction of daphnids which survived to reproduce at low pH did not appear significantly affected by pH.

In this study, daphnids produced smaller neonates at pH 5.7 and 7.7. The reduced neonate size at pH 5.7 might be related to both reduced mother size (Ebert 1993) and reduced energy available for reproduction as a consequence of high maintenance costs. On the other hand, the reduced neonate size at pH 7.7 might be related to improved feeding rate (Ebert 1993) as illustrated in Figure 3.6. Under good food conditions, daphnids tend to produce more and smaller neonates (Ebert 1993). The neonate size affect the number of instars to maturity: reduced neonate size decreases age and size at maturity and, thus, fertility at first

reproduction (Ebert 1994). Moreover, reduced neonate size might possibly diminish their tolerance to acidic stress. Enserink and coworkers (1990) showed that smaller neonates are more sensitive than larger ones to cadmium and a similar situation was found for environmental stresses such as starvation and oxygen deficiency (Hanazato and Dodson 1995).

The relative feeding rate of daphnids (both juveniles and adults) registered higher values at pH 7.7, which follows the known "rule" that cladocerans have maximal feeding rates under conditions similar to those under which they were cultured (Kring and O'Brien 1976). The reduced feeding rates at high pH are concordant with previous findings (e.g. O'Brien and DeNoyelles Jr. 1972). However, this might be indicative that daphnids used in the feeding inhibition tests were not completely acclimated to the test conditions, although they were exposed to the test conditions for the entire developmental period, as suggested by Barata and coworkers (2007). The same applies to daphnids used in the chronic tests (21 d), since they were exposed to the same acclimation period. If the daphnids were not fully acclimated to the test conditions, they might have exhibited more pronounced responses than acclimated daphnids, for instance, lower feeding rates (Kring and O'Brien 1976) and lower reproduction (Lasier, Winger et al. 2006).

The results on the feeding rates of juveniles and adults are in relative accordance with the neonate size, BL at day 21 and cumulative fertility, thus presenting evidence of the ecological relevance of feeding rates (McWilliam and Baird 2002). Feeding rate determines energy acquisition and further allocation, thus affecting life-history traits of *Daphnia* (Nogueira, Baird et al. 2004) and, consequently, affecting population growth. Indeed, daphnids with lower feeding rates produced fewer offspring and, consequently, exhibited lower intrinsic rate of population growth (Walton, Compton et al. 1982; Hooper, Connon et al. 2008).

We found that pH 5.7 was detrimental for *D. magna* by reducing survival, which supports a previous study that states that *Daphnia* species are not found at pH values below 5.8 (Almer, Dickson et al. 1974). However, several authors reported other pH values as critical for *Daphnia* species (e.g. Parent and Cheetham 1980; Walton, Compton et al. 1982). Thus, we should look at pH values in this study as "guidance" values for two main reasons. First, the acid-tolerance varies within *Daphnia* species; for instance, *D. middendorffiana* is more

acid-tolerant than *D. pulex* and *D. magna* (Locke 1991) and, due to genetic variability, it might also vary among clones of the same species as already reported with toxic chemicals (Barata, Baird et al. 1998). Second, the acid-tolerance is affected by other water chemical parameters, namely hardness, as shown in this study. Increasing hardness frequently reduced H^+ toxicity to daphnids (Locke 1991), most pronouncedly the increase from the low to the high level (0.5 to 1.7 mM). The further increase in hardness (1.7 to 3.5 mM, approximately) tended to dissipate the effects of pH improving the performance of daphnids reared at low pH and reducing it in daphnids reared at high pH, pointing to an interaction between pH and hardness, concordantly to previous studies (Locke 1991; Glover and Wood 2005; Hooper, Connon et al. 2008). The interaction between pH and hardness can play an important role in aquatic ecosystems, namely concerning the extinction of *Daphnia* populations. Although the extinction of *Daphnia* populations is usually attributed to decreasing Ca concentration (Jeziorski, Yan et al. 2008), it might be, indeed, a consequence of decreased Ca concentration allied to low pH since we found that daphnids could survive at low hardness but not simultaneously at low hardness and low pH. Thus, the effects of varying water pH on life-history traits of *Daphnia* and other acid-sensitive crustaceans cannot be assessed independently of the hardness level.

The consequences of the hardness-mediated amelioration of H^+ toxicity are important in laboratorial assays since most assays are conducted using hard water (Locke 1991), following recommendations from international organizations (e.g. OECD 2008). Thus, some species may be more acid-sensitive than suggested by bioassays conducted in hard water. Given the high correlation between hardness and alkalinity in natural waters (Okland and Okland 1986), hard water lakes frequently have high alkalinity, which prevents pH changes.

Water acidification is an important stressor in lake ecosystems affecting the distribution and structure of zooplankton communities through several mechanisms. As proposed by Weber and Pirow (2009), these mechanisms include a differential physiological sensitivity to acid stress (Locke 1991; Havens, Yan et al. 1993), a differential sensitivity to toxic metal species which become more soluble under acidic conditions (Lawrence and Holoka 1987; Havens 1990), and also altered biotic interactions arising from the effects of reduced pH on phytoplankton and planktivorous communities (Locke and Sprules 2000). The effects on

phytoplankton include changes in taxonomic composition, abundance and quality (Locke and Sprules 2000). By affecting the structure of aquatic food webs, these mechanisms can cause severe effects not only to zooplankton communities but also to potentially all aquatic organisms in the ecosystem. Additionally, aquatic organisms may be exposed to seasonal variations in pH, such as spring pH depressions (Locke 1991), with consequences for their life history.

In summary, this study showed the central role of pH to life-history traits and population growth of *Daphnia* and suggested an interaction between pH and hardness. The effect of pH was more adverse at low hardness levels, which raises concern about the effects of acid deposition to *Daphnia* and, consequently, to the structure of aquatic food webs.

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CHAPTER

4

COMBINED EFFECTS OF TEMPERATURE AND WATER CHEMISTRY ON LIFE-HISTORY TRAITS AND POPULATION GROWTH OF *DAPHNIA MAGNA*

4. Combined effects of temperature and water chemistry on life-history traits and population growth of *Daphnia magna*

Abstract

We exposed *Daphnia magna* to several combinations of water hardness (0.46 to 3.45 mM) and alkalinity (0.32 to 2.27 mM) at 13, 15, 20, 25 and 30ºC, and evaluated the effects on growth, reproduction and population growth. Temperature was crucial for *D. magna*, affecting all the studied endpoints. In general, extreme temperatures reduced growth, reproduction and, consequently, intrinsic rate of population growth of the daphnids. Water hardness and alkalinity also caused significant effects to *Daphnia*, but these effects were dependent on temperature, as they were more pronounced at 13, 20 and 30ºC with little variability at 15 and 25ºC. Moreover, daphnids showed reduced body length and cumulative fertility at high temperatures, suggesting that higher temperatures increase the susceptibility of daphnids to low hardness and alkalinity. Furthermore, the relationship body length - brood size was affected by temperature, showing reduced slopes at extreme temperatures.

This study presents evidences that increasing temperature up to 25ºC or more causes adverse effects on life-history traits of *Daphnia* and increases their susceptibility to low hardness and alkalinity, which can have cumulative effects at long-term. Similarly, a decrease in temperature below 15ºC will likely have negative effects for *Daphnia* populations.

Keywords: temperature; water chemistry; *Daphnia magna*; growth; reproduction; population growth

4.1. Introduction

The distribution of organisms is conditioned by abiotic factors that affect the physiological processes and life-history traits of individuals and, thus, persistence and growth of populations. Among abiotic factors, temperature is one of the most important since about

99.9% of species on Earth are ectothermic, that is, they rely primarily on external sources for their body heat (Atkinson and Sibly 1997). Moreover, climate change has raised concern about the effect of varying temperature on the Earth's ecosystems.

Aquatic organisms are particularly exposed to the effects of varying temperature. In the aquatic environment most of the organisms, including fish and all invertebrates, are ectotherms and, thus, highly dependent on water temperature. The effects of temperature are particularly pronounced in species living in shallow-water ecosystems (ponds, pools, marshes) since temperature variations are not buffered by the presence of a large water mass (Gordo, Lubian et al. 1994) and, thus, those species are exposed to wide diurnal and seasonal temperature variations. Moreover, at extreme temperatures aquatic organisms will have to face the eventual oxygen shortage in their bodies. An increase in water temperature increases metabolic activity and, thus, oxygen demand of aquatic organisms (Mourelatos and Lacroix 1990). However, when exposed to increasing water temperature, aquatic organisms also have to face the subsequent decrease of oxygen solubility in the water. In opposition, at low temperatures, although oxygen demand is reduced, the remaining energy metabolism may not be sufficiently supplied with oxygen because of the cessation of ventilation and perfusion and the muscular activity necessary for these processes (Zeis, Maurer et al. 2004).

Temperature affects the rate of most physiological processes, thus, affecting growth, development, reproduction, respiration, behavior and, ultimately, survival of aquatic organisms, namely *Daphnia* (Goss and Bunting 1983; Mourelatos and Lacroix 1990; Koh, Hallam et al. 1997; Chopelet, Blier et al. 2008). Ectotherms reproduce later and at a larger size and grow to bigger final sizes when reared at lower temperatures, which is known as the "temperature-size rule" (Atkinson 1994; Atkinson and Sibly 1997),

However, aquatic systems are exposed not only to changes in water temperature, but also to changes in the water chemistry parameters. A reduction of water hardness has been reported in many locations over Europe and North America (Skjelkvåle, Stoddard et al. 2005) which is mainly associated with the decreased concentration of Ca from surrounding soils (Watmough, Aherne et al. 2005). This phenomenon is partly attributed to acid deposition, which also causes decreased water pH and alkalinity. Thus, aquatic systems are

simultaneously affected by varying temperature and water chemistry, namely hardness and alkalinity.

In this work we selected the species *Daphnia magna* as representative of freshwater crustaceans. This species is mainly found in eutrophic ponds, rock pools and shallow lakes (Hebert 1978; Lampert 1991). These small-scale systems are particularly exposed to variations of temperature and water chemistry since those variations are not buffered by the presence of a large water mass.

Life-history traits and population growth of *D. magna* are affected by water hardness and alkalinity, as shown previously. Moreover, *Daphnia* become more susceptible to low Ca concentrations at increasing temperatures, as shown by Ashforth and Yan (2008). These authors studied temperatures above 20ºC and Ca concentrations below 0.25 mM. In this paper we studied a larger temperature range (13 – 30ºC) and water chemistry (Ca concentrations between 0.18 and 1.66 mM). We hypothesized that temperature would affect the responses of *D. magna* to varying water chemistry parameters hardness and alkalinity. Hence, this study was undertaken to assess the combined effects of varying temperature and water chemistry on growth, reproduction and intrinsic rate of population growth of *D. magna*. The temperature range 13-30ºC was selected as representative for *Daphnia* as, in the absence of toxicants, *Daphnia* populations persist within the temperature range from 11.0 to 30.6ºC (Koh, Hallam et al. 1997). To represent natural waters we selected 6 media with varying levels of hardness (0.5 to 3.5 mM as $CaCO_3$) and alkalinity (0.3 to 2.3 mM as $CaCO_3$).

D. magna was selected as a model test species for several reasons. First, *Daphnia* are widely distributed ectotherms, inhabiting lentic waters around the world, mainly lakes and ponds, thus especially vulnerable to the effects of varying temperature. Second, *Daphnia* are amongst the aquatic species more vulnerable to water hardness, given their high Ca demand (Jeziorski and Yan 2006). And finally, *Daphnia* are representative of filter-feeding zooplankton, which have a major contribution to aquatic food webs in many freshwaters. Thus, if major changes occur within these communities there is potential of wide impacts in aquatic food webs.

4.2. Material and Methods

4.2.1. Test organisms

Parent organisms originated from a single clone of *Daphnia magna* Straus (1820) clone F (sensu Baird, Barber et al. 1990). Organisms were individually cultured in 100 ml of ASTM hard water (ASTM 2004) with a standard organic additive (Marinure seaweed extract, supplied by Glenside Organics Ltd.) and fed *Chlorella vulgaris* (5.5 µg dw/ml/day) which were cultured in MBL medium. Culture medium was renewed every other day. Temperature was 20 ± 1ºC and photoperiod was 16 h light: 8 h dark.

Parental daphnids were acclimated to the test conditions (temperature and medium) before releasing the first brood. Acclimation to test temperature was performed gradually, in order to minimize impacts on the organisms. The first two broods were discarded. Only neonates from third or fourth broods were used in the experiments, as these organisms were exposed to the test conditions during their entire developmental period (Barata and Baird 2000).

4.2.2. Experimental design

Six synthetic media with varying levels of hardness and alkalinity were selected for testing, having in consideration that in natural waters there is a significant degree of covariability between hardness and alkalinity (Okland and Okland 1986). Media were prepared by adding different volumes of stock solutions of $CaSO_4.2H_2O$, $MgSO_4.7H_2O$, $NaHCO_3$ and KCl (analytical-grade) to Milli-Q water. Hardness levels were low (L), moderate (M), high (H) and very high (VH), corresponding to 0.5, 0.9, 1.7 and 3.5 mM as $CaCO_3$, respectively. Alkalinity levels were low (L), moderate (M), high (H) and very high (VH), corresponding to 0.3, 0.6, 1.1 and 2.3 mM as $CaCO_3$, respectively. For clarity, media are coded as levels of "hardness.alkalinity". For instance, medium M.H refers to a medium with moderate hardness (0.9 mM) and high alkalinity (1.1 mM). Though hardness and alkalinity are commonly expressed as concentration of $CaCO_3$, for simplicity, we will omit "as $CaCO_3$" hereafter. The chemical properties of the test media are described in Table 4.1. New media were prepared every other day, stored at the test temperature and used to renew daphnids' media daily.

Table 4.1: Chemical properties of the test media: mean (SD), n≥12. All measurements were done at 20ºC

medium	hardness (mM as $CaCO_3$)	alkalinity (mM as $CaCO_3$)	conductivity ($\mu S\ cm^{-1}$)	pH	concentration of major ions (mM)					
					Ca	Mg	Na	K	Cl	SO4
L.L	0.46 (0.02)	0.32 (0.02)	158.3 (16.9)	7.85 (0.21)	0.18 (0.01)	0.26 (0.02)	0.67 (0.06)	0.11 (0.01)	0.08 (0.02)	0.60 (0.04)
M.M	0.90 (0.04)	0.58 (0.03)	291.6 (31.0)	8.08 (0.18)	0.37 (0.03)	0.51 (0.05)	1.25 (0.12)	0.13 (0.01)	0.10 (0.02)	1.10 (0.07)
M.H	0.89 (0.04)	1.12 (0.06)	389.4 (41.2)	8.34 (0.14)	0.37 (0.03)	0.51 (0.05)	2.41 (0.25)	0.19 (0.02)	0.16 (0.02)	1.12 (0.08)
H.L	1.75 (0.06)	0.33 (0.02)	402.3 (39.7)	7.92 (0.21)	0.79 (0.08)	1.03 (0.12)	0.69 (0.08)	0.20 (0.02)	0.16 (0.02)	2.07 (0.10)
H.H	1.75 (0.06)	1.14 (0.06)	546.4 (50.5)	8.34 (0.14)	0.76 (0.05)	1.00 (0.11)	2.44 (0.23)	0.20 (0.02)	0.16 (0.02)	2.08 (0.11)
VH.VH	3.45 (0.10)	2.27 (0.12)	982.8 (92.6)	8.43 (0.16)	1.66 (0.22)	1.98 (0.24)	4.71 (0.52)	0.31 (0.03)	0.25 (0.04)	4.09 (0.22)

4.2.3. Experimental procedure

Tests were initiated with neonates originated from parental daphnids acclimated to the experimental conditions (water chemistry and temperature), using twenty replicates per medium (N=600). Organisms were individually reared in glass vials containing 100 ml of test medium during 21 days and evaluated for growth and reproduction. The algal and seaweed extract concentrations, as well as the photoperiod and temperature conditions were as described for culturing. Age at successive growth or reproductive events was measured with 24h accuracy (experiments at 15 and 20ºC), 12h (experiments at 13 and 25ºC) or 8h (experiment at 30ºC). Test media were renewed daily. During the media renewal all live offspring and aborted eggs and embryos in each vial were counted and discarded. Additionally, the shedded carapaces of test organisms were collected and used to determine the body length (BL, from head to the base of spine, mm), thus avoiding stressing test organisms. BL was estimated based on the length of the first exopodite of the second antennae (AL, mm) measured from daphnids' shedded carapaces. The following equation was used: $BL = 10.98 \times AL - 0.55$ (r^2=0.978, n=128, p<0.0001). AL was measured under a stereomicroscope (MS5, Leica Microsystems, Houston, TX, USA) with a calibrated eyepiece micrometer.

The combined effects of temperature and water chemistry on growth and other developmental endpoints were assessed by determining the following endpoints: body length (BL) at hatching, BL at day 7, BL at day 14, BL at day 21, number of juvenile instars (number of instars daphnids go through before eggs are first observed in the brood chamber) and adult instar duration.

To assess reproductive effects several life-history traits were evaluated: age at first reproduction (AFR, age of the daphnid when the first brood is released); size at first reproduction (SFR, length of the daphnid when carrying the first brood in the brood chamber); fertility at first reproduction (FFR, number of viable juveniles produced in the first reproduction) and cumulative fertility (number of viable juveniles released during the 21 days period). We also assessed whether the relationship body length - brood size (viable offspring per brood), a "standard" relationship in daphnids reproduction (Green 1956; Nogueira, Baird et al. 2004), was affected by temperature or water chemistry.

4.2.4. Chemical analyses

Concentration of major ions was measured on random samples collected throughout the test period, both fresh and 24h-old media (i. e. before and after renewal of the test media) after filtration (0.45µm cellulose acetate membrane). Concentration of anions was measured in a Hach DR2000 spectrophotometer (Germany): chloride was determined by the mercuric thyocianate method and sulfates by the Sulfaver 4 method. Concentration of cations (Ca, Mg, Na and K) was determined in samples acidified with concentrated nitric acid, using inductively coupled plasma mass spectrometry (ICP-MS Thermo Scientific X-Series).

Total hardness and total alkalinity were measured using the EDTA and the bromocresol green titrimetric procedures, respectively (American Public Health Association 2005).

Additionally, conductivity, dissolved oxygen, pH and temperature were measured daily, using a WTW Cond 330i meter, a WTW Oxi 330 meter and a WTW pH 330 meter, respectively.

4.2.5. Data treatment and statistical analyses

Growth data was fitted to the model of somatic growth presented by Rinke and Petzoldt (2003) which is based on the von Bertalanffy equation. These authors developed the equation for the *Daphnia galeata/hyalina* species complex, so it contained appropriate functional relationships, but an inadequate parameterization. Thus, we determined new parameters, specifically for *Daphnia magna*.

Although the effects of water chemistry on somatic growth were found to be statistically significant, they were very small compared to those of temperature. Thus, we opted to describe the somatic growth only as a function of temperature – this approach allowed the description of the main variability in daphnids length (temperature effects) keeping the model relatively simple.

The model used to describe the somatic growth of daphnids at varying temperatures is shown in equation 4.1, as presented by Rinke and Petzoldt (2003):

$$L = L_{\max} - (L_{\max} - L_0)e^{-kt} \quad \text{(equation 4.1)}$$

whereat:

$$k = k_1 e^{k_2 T} \quad \text{(equation 4.2)}$$

with

L = body length (mm)

L_{max} = maximal body length (mm)

L_0= body length at hatching

t = age (d)

T = temperature (°C).

k_1, k_2= constants.

We observed that both L_{max} and L_0 varied with temperature, following a maxima function, as illustrated in equations 4.3 and 4.4.

$$L_{max} = k_3 \times T^{k_4} \times e^{k_5 T} \quad \text{(equation 4.3)}$$

$$L_0 = k_6 \times T^{k_7} \times e^{k_8 T} \quad \text{(equation 4.4)}$$

where

k_3, k_4, k_5, k_6, k_7, k_8 = constants

An overall parameter estimation (least squares) was conducted on the complete dataset.

Effects at the population level were assessed with the intrinsic rate of population growth (r) according to the Euler-Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x \quad \text{(equation 4.5)}$$

where x is the age in days (0...n), l_x is the age specific survivorship and m_x is the number of newborns on day x.

To assess the statistical significance of the combined effects of temperature, water chemistry and their interaction on growth, reproduction and intrinsic rate of population growth of *D. magna*, we employed a two-way ANOVA following a General Linear Model procedure (Minitab 15, Minitab Inc., Pennsylvania, USA) and multiple comparisons were performed with the Tukey´s test. Normality of the data was checked by the Ryan-Joiner and homogeneity of variances by examination of residuals. Heteroscedastic variables or with a

non-normal distribution were analyzed with the nonparametric test Kruskal-Wallis and the Dunn's post-hoc test (SigmaPlot, v. 10, Systat Software Inc.).

Analysis of Covariance (ANCOVA) was performed in order to determine if the relationship body length - brood size (viable offspring per brood) was affected by temperature or water chemistry.

All statistical analyses were based on 0.05 significance level.

4.3. Results

As expected, the dissolved oxygen (DO) concentration decreased with increasing temperature. DO (mean ± SD) varied between 7.58 ± 0.30 mg/L at 30ºC and 9.31 ± 0.73 mg/L at 13ºC.

Temperature was found to be determinant for growth, reproduction and intrinsic rate of population growth of *D. magna*, affecting all the studied endpoints (Table 4.2). The effect of water chemistry and the interaction between temperature and water chemistry was significant for the majority of the studied endpoints. The interactive effects of temperature and water chemistry are evidenced by the fact that major differences among media were observed at 20ºC and extreme temperatures (13 and 30ºC). In opposition, at 15 and 25ºC, water chemistry played a minor role, causing significant variability only in a few endpoints.

At high temperatures (25 and 30ºC), medium L.L proved to be unfavorable for daphnids, causing reduced BL at day 21 and reduced cumulative fertility; this was more evident at 30ºC, as more endpoints were affected, namely increased production of non-viable offspring and reduced intrinsic rate of population growth. Media H.H and VH.VH, those with higher hardness and alkalinity, caused the opposite effects. On the other hand, at temperatures equal or below 20ºC we did not observe clear evidence of reduced growth and/or reproduction of daphnids in medium L.L.

Only the effects on the most relevant endpoints are illustrated graphically; more information on these and other endpoints are presented in Table 4.5 (Supplementary material).

Table 4.2: Probability values for the statistical testing (two-way ANOVA) of the effects of temperature, water chemistry and their interaction on life-history traits of *D. magna*. SFR: age at first reproduction; SFR: size at first reproduction; FFR: fertility at first reproduction

	endpoints	significant covariates	temperature	water chemistry	interaction
growth and development	BL at day 0	---	< 0.001	**0.059**	**0.099**
	BL at day 7	---	< 0.001	< 0.001	< 0.001
	BL at day 14	---	< 0.001	0.001	< 0.001
	BL at day 21	---	< 0.001	< 0.001	< 0.001
	number of juvenile instars	---	< 0.001	< 0.001	< 0.001
	adult instars duration	---	< 0.001	**0.489**	**0.775**
reproduction	AFR	---	< 0.001	< 0.001	< 0.001
	SFR	---	< 0.001	< 0.001	< 0.001
		age (p<0.001)	< 0.001	< 0.001	< 0.001
	FFR	---	< 0.001	< 0.001	< 0.001
		SFR (p<0.001)	< 0.001	**0.247**	< 0.001
	cumulative fertility	---	< 0.001	< 0.001	< 0.001
		AFR (p<0.001)	< 0.001	< 0.001	< 0.001
	number of broods	---	< 0.001	< 0.001	< 0.001
	average brood size	---	< 0.001	0.016	**0.499**
	non-viable offspring	---	< 0.001	< 0.001	< 0.001
population	intrinsic rate of population growth, *r*	---	< 0.001	< 0.001	< 0.001

Growth and development

Body length of the daphnids at hatching, day 7, day 14 and day 21 are shown in Figure 4.1. These endpoints were affected by temperature, water chemistry and its interaction, with the exception of BL at day 0 which was only dependent on temperature. BL at day 0 decreased

with increasing temperature, thus neonates at 13ºC and 15ºC were the largest. However, from day 7 onwards daphnids reared at 20ºC were the largest. Daphnids at 30ºC were the smallest from day 14 onwards, due to the strong reduction in their body length during this period. In general, the body length of daphnids reared at 13 and 15ºC did not differ among each other, which might be related to the small difference between these temperatures: 2ºC.

As referred previously, the effects of water chemistry on somatic growth, although statistically significant, were diminutive compared to those of temperature. The somatic growth of daphnids between days 0 and 21 days could, thus, be described based only on temperature. Parameters estimation is presented in Table 4.3, and resulted in a high coefficient of determination (Figure 4.2, r^2=0.971, n=4986). Figure 4.2 shows that increasing temperature causes an increased curvature of the growth model which corresponds to a smaller maximum body length (BL_{max}).

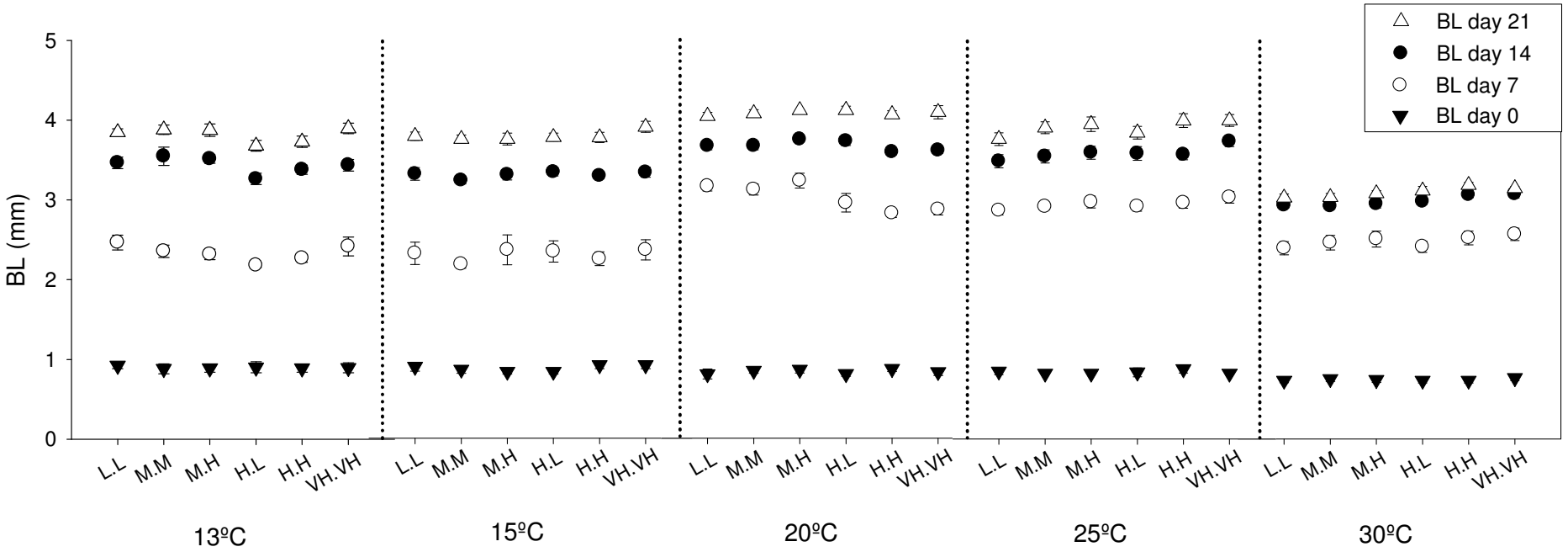

Figure 4.1: Combined effects of temperature and water chemistry on growth endpoints of *D. magna*: BL at day 0, BL at day 7, BL at day 14 and BL at day 21. Means and 95% confidence intervals are represented. Results of multiple comparisons among media are presented in Table 4.5 (Supplementary material)

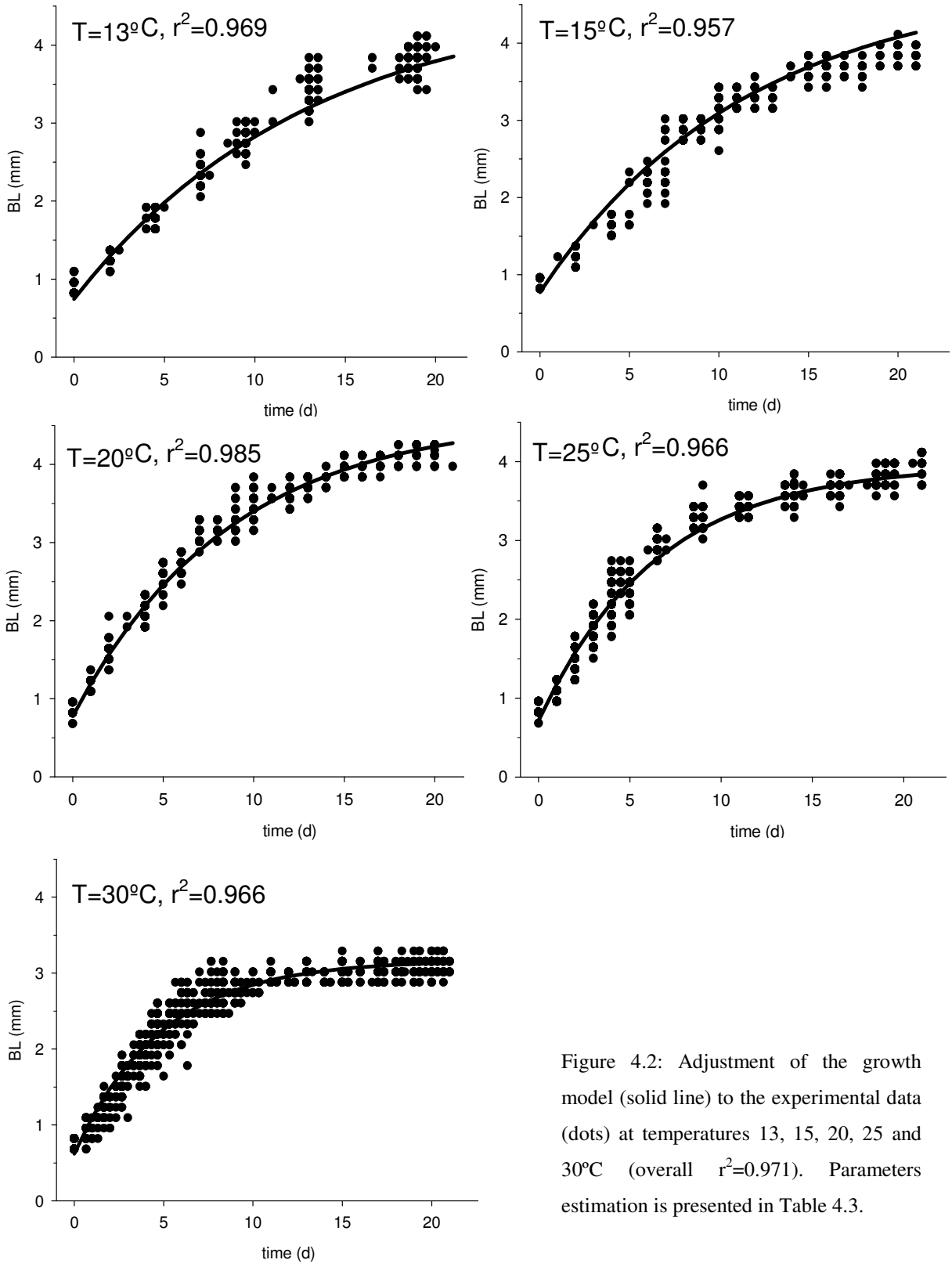

Figure 4.2: Adjustment of the growth model (solid line) to the experimental data (dots) at temperatures 13, 15, 20, 25 and 30ºC (overall r^2=0.971). Parameters estimation is presented in Table 4.3.

Table 4.3: Parameter estimates for the multiple regression model of somatic growth, using equations 4.1 - 4.4

parameter	value	Unit
k_1	0.036	d^{-1}
k_2	0.058	$(ºC)^{-1}$
k_3	0.210	mm $(ºC)^{-1}$
k_4	1.758	---
k_5	-0.109	$(ºC)^{-1}$
k_6	0.064	mm $(ºC)^{-1}$
k_7	1.348	---
k_8	-0.077	$(ºC)^{-1}$

The number of juvenile instars varied between 4.0 and 5.0 and was affected by temperature, water chemistry and their interaction (Table 4.2; Table 4.5). In general, the number of juvenile instars decreased with increasing temperature, but registered an increase between 25 and 30ºC. Water chemistry caused significant differences only at 20 and 30ºC: increasing hardness and alkalinity caused the number of juvenile instars to decrease. This variation is particularly noticeable at 20ºC: daphnids reared in media L.L and M.M had 5.0 juvenile instars, whereas daphnids reared in media H.H and VH.VH had only 4.1 juvenile instars.

Adult instar duration was only affected by temperature (Table 4.2, Table 4.5), showing a decrease with increasing temperature.

Reproduction

The reproduction of *D. magna* was highly dependent on water temperature. The vast majority of the studied endpoints differed among all temperatures, including between 13 and 15ºC suggesting that at extreme temperatures, even small variations, such as 2ºC, may significantly affect the reproduction of *Daphnia*. Note that, in contrast, concerning growth endpoints, only BL at day 14 and the duration of adult instars differed between temperatures 13 and 15ºC.

The combined effects of temperature and water chemistry on the reproduction life-history traits are presented in Figures 4.3 to 4.6 and in Table 4.5. AFR decreased with increasing temperature (Figure 4.3), which is in accordance with the variation in the number of juvenile instars described previously. The effects of water chemistry were significant only at 20ºC: the increase of hardness and alkalinity caused the AFR to decrease, concordantly to the variation observed in the number of juvenile instars.

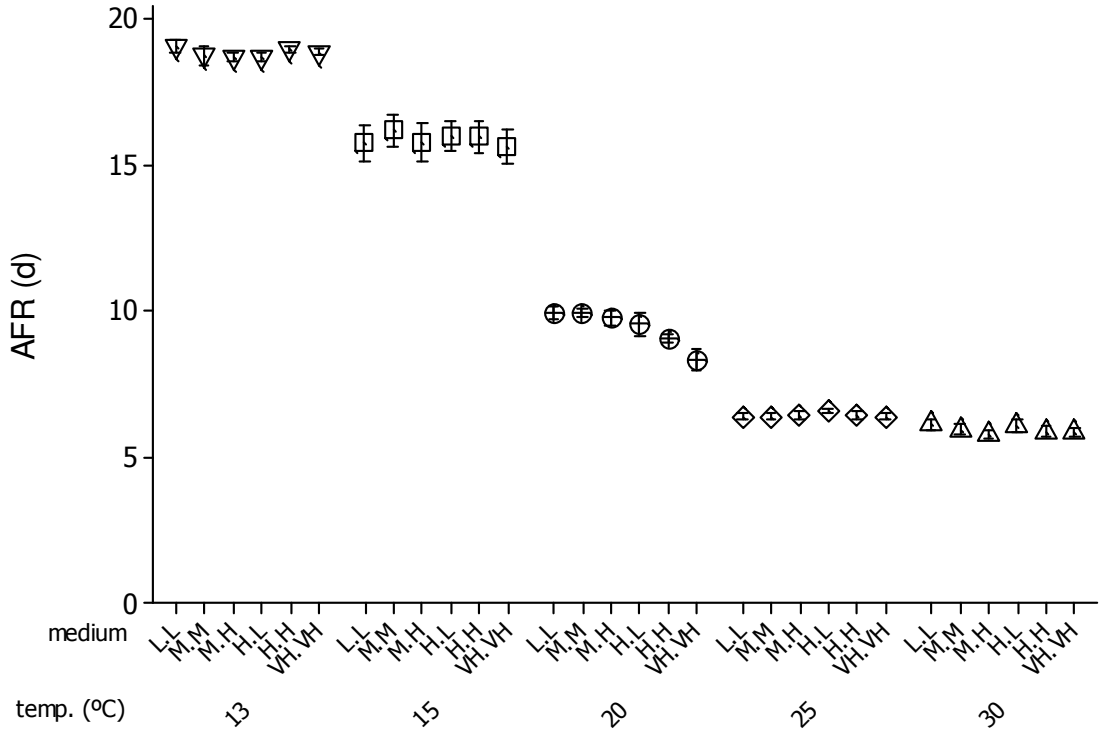

Figure 4.3: Combined effects of temperature and water chemistry on age at first reproduction (AFR) of *D. magna*. Symbols represent means and vertical bars represent 95% confidence intervals. Results of multiple comparisons among media are presented in Table 4.5 (Supplementary material).

As AFR, also SFR was reduced with increasing temperature (Figure 4.4). The effect of water chemistry was dependent on temperature: at 13 and 20ºC increasing hardness reduced SFR, but an opposite trend was observed at 30ºC. The variations in SFR among temperatures are partly attributed to AFR (Table 4.2).

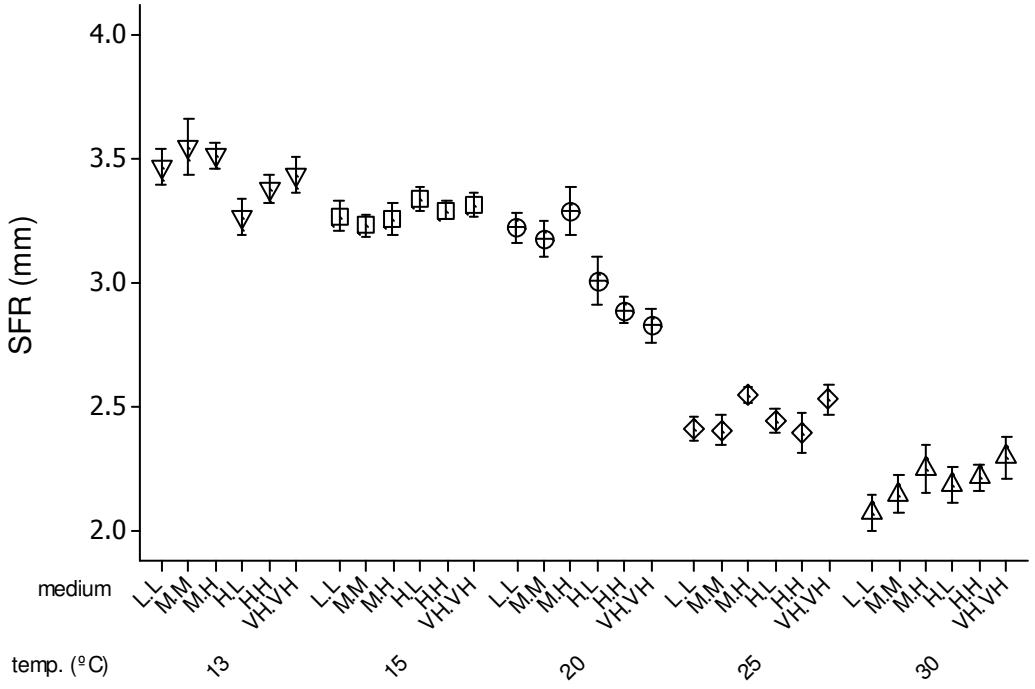

Figure 4.4: Combined effects of temperature and water chemistry on size at first reproduction (SFR) of *D. magna*. Symbols represent means and vertical bars represent 95% confidence intervals. Results of multiple comparisons among media are presented in Table 4.5 (Supplementary material).

Concerning FFR (Figure 4.5), it varied with temperature following a bell-shaped curve with the maximum at 15ºC. Since larger *Daphnia* produce more offspring, we would expect daphnids at 13ºC to have the highest FFR, which was not the case. At 13ºC although larger, daphnids did not produce more offspring, which is valid also considering the total offspring (i.e., total investment in the first brood) instead of viable offspring in the first brood (FFR). Concerning water chemistry, there is no direct effect on FFR, but an indirect effect through variation in SFR (Table 4.2).

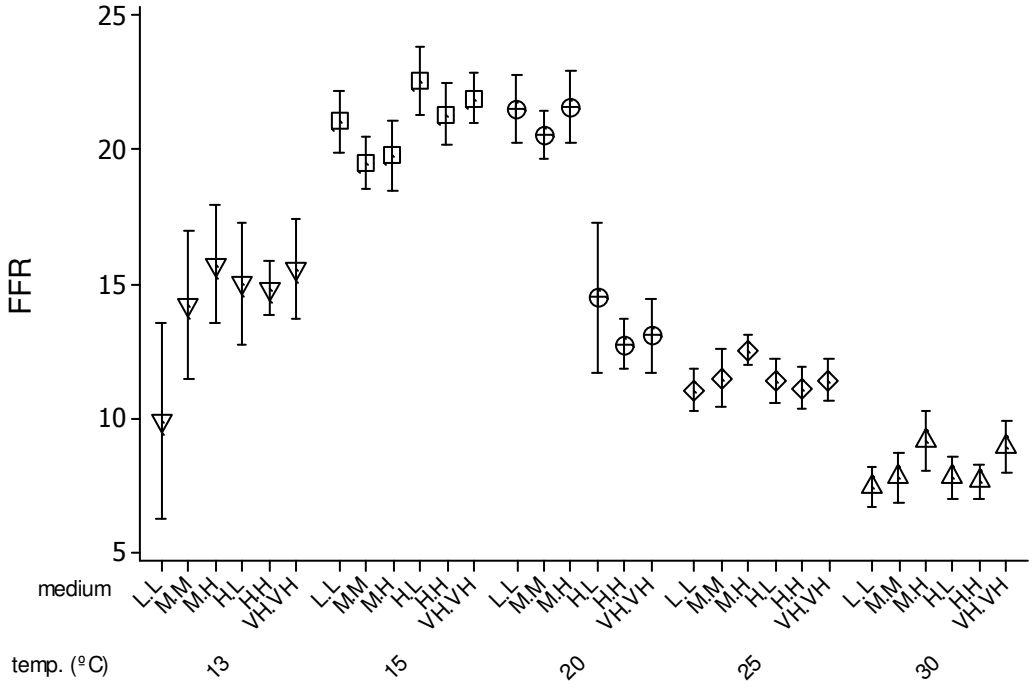

Figure 4.5: Combined effects of temperature and water chemistry on fertility at first reproduction (FFR) of *D. magna*. Symbols represent means and vertical bars represent 95% confidence intervals. Results of multiple comparisons among media are presented in Table 4.5 (Supplementary material).

Cumulative fertility (Figure 4.6), like FFR, varied with temperature following a bell-shaped curve, but with a maximum at 25ºC. At low temperatures (13 and 15ºC) water chemistry did not affect this endpoint but at temperatures equal or above 20ºC we observed increased cumulative fertility with increasing hardness and alkalinity. However, the fact that water chemistry did not cause significant differences at low temperatures (13 and 15ºC) might not be conclusive. Actually, at these temperatures less than 2 broods per daphnid (in average) were produced (Table 4.5), whereas at higher temperatures the production of more broods might have enhanced the differences among test media, resulting in a significant effect of water chemistry.

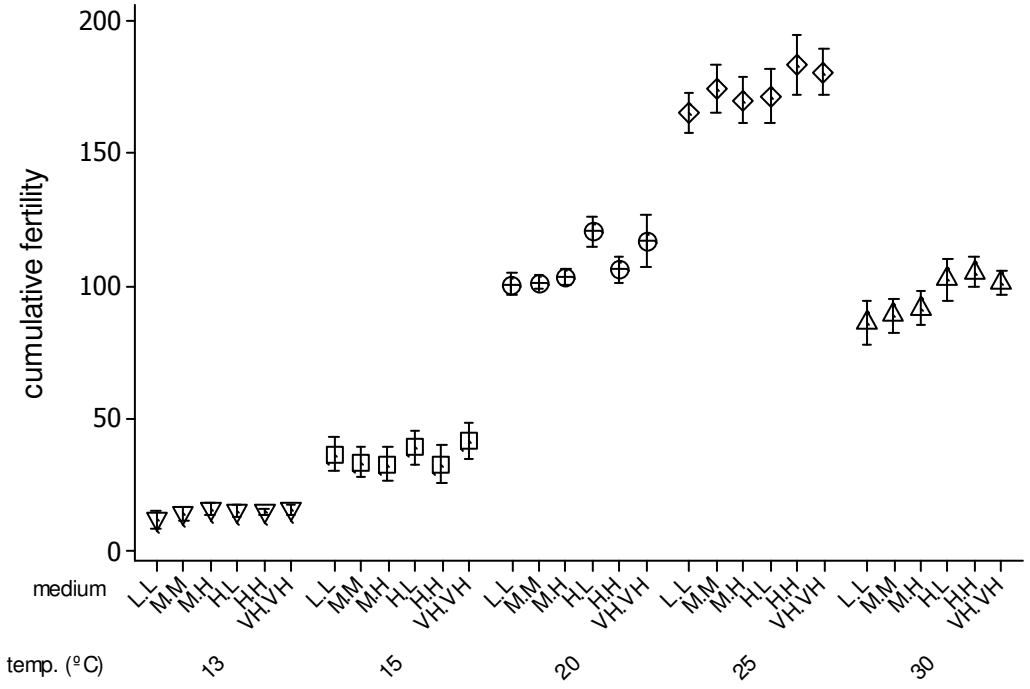

Figure 4.6: Combined effects of temperature and water chemistry on cumulative fertility of *D. magna*. Symbols represent means and vertical bars represent 95% confidence intervals. Results of multiple comparisons among media are presented in Table 4.5 (Supplementary material).

The number of broods increased with temperature (Table 4.5), which is related to both reduced AFR and decreased adult instar duration with increasing temperature.

Like cumulative fertility, the average brood size also followed a bell-shaped curve with a maximum at 25ºC (Table 4.5).

Extreme temperatures affected the developmental success of eggs and embryos, resulting in non-viable offspring (aborted eggs and/or dead embryos or juveniles) (Table 4.5). At 13ºC all test media caused the production of non-viable offspring, showing a clear dependence on water chemistry: as hardness and alkalinity decrease the number of non-viable offspring increases. Concordantly, at 30ºC, although all test media caused the production of non-viable offspring, this effect was more pronounced in daphnids reared in medium L.L.

The relationship body length - brood size of daphnids was affected by temperature (Figure 4.7A; Table 4.4). Within our data range this relationship was well described by a linear

function. Analysis of covariance showed that regression lines for the different test media are not coincidental ($F_{8;1978}$=276.11; p<0.0001). Significant differences among slopes were found ($F_{4;1978}$=172.18; p<0.0001): 30ºC=13ºC=15ºC≠20ºC=25ºC. However, the regression line developed for the temperature 13ºC was not significant (Table 4.3), most probably due to the small range of body length data. Nevertheless, our results suggest that adult daphnids with the same body length will produce smaller broods at 15 or 30ºC than at 20 or 25ºC. The effect of water chemistry on the relationship body length - brood size was significant only at 20ºC: L.L=M.M≠H.L. This means that daphnids tended to produce smaller broods if reared in media with low to moderate hardness and alkalinity.

Table 4.4: Results of the ANCOVA analysis on the effects of temperature on the relationship body length - brood size of *D. magna*. N.S.: not statistically significant

	13ºC	15ºC	20ºC	25ºC	30ºC
slope	2.477	7.240	17.015	17.242	0.447
r^2	0.006	0.226	0.753	0.812	0.153
n	93	182	398	537	777
p	N.S.	<0.0001	<0.0001	<0.0001	<0.0001

Daphnids reared at 30ºC produced smaller broods at any instar (Figure 4.7B). The largest broods were produced around the 8^{th}-10^{th} instars (temperatures 20, 25 and 30ºC), after which a slight trend for decrease was observed (temperatures 25 and 30ºC).

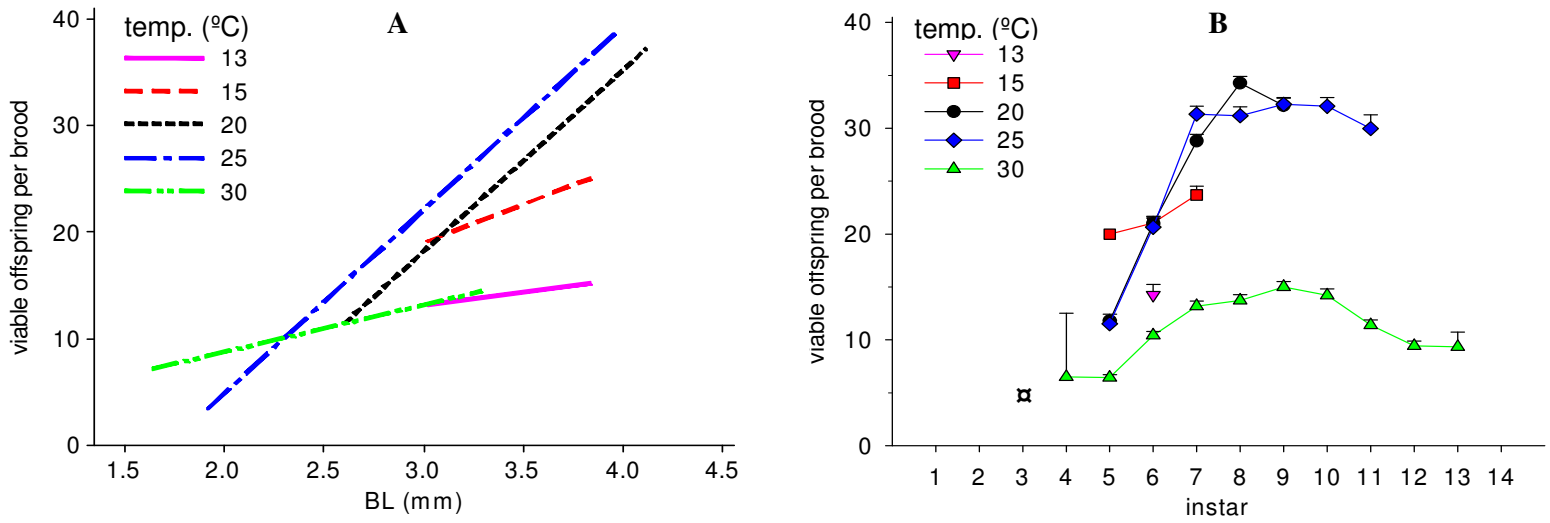

Figure 4.7: Brood size (viable offspring per brood) as a function of (A): body length (relationship body length - brood size) and (B): instar (brood size in each instar) at different temperatures (13, 15, 20, 25 and 30ºC).
¤: at 30ºC only two daphnids (in 120) released offspring at the end of the 4th instar; the remaining released the first brood in the 5th or 6th instars.

Intrinsic rate of population growth

The combined effects of temperature and water chemistry on the intrinsic rate of population growth (*r*) are illustrated in Figure 4.8. This endpoint is affected by the temperature, following a second degree polynomial function: $r = -0.68 + 0.081T - 0.0014\ T^2$ (n=605; r^2-adj = 0.959; $p<0.001$) where T represents temperature (ºC). Following this, *r* is expected to be maximal at 28.4ºC. Although water chemistry significantly affected *r*, the inclusion of hardness and/or alkalinity in this regression decreased the model fit. Increasing hardness and alkalinity increased *r*, most clearly at 15 and 30ºC. The interaction between temperature and water chemistry on *r* can be illustrated by the fact that increasing the temperature from 25 to 30ºC causes a reduction in *r*, but only for media with low to moderate hardness and alkalinity.

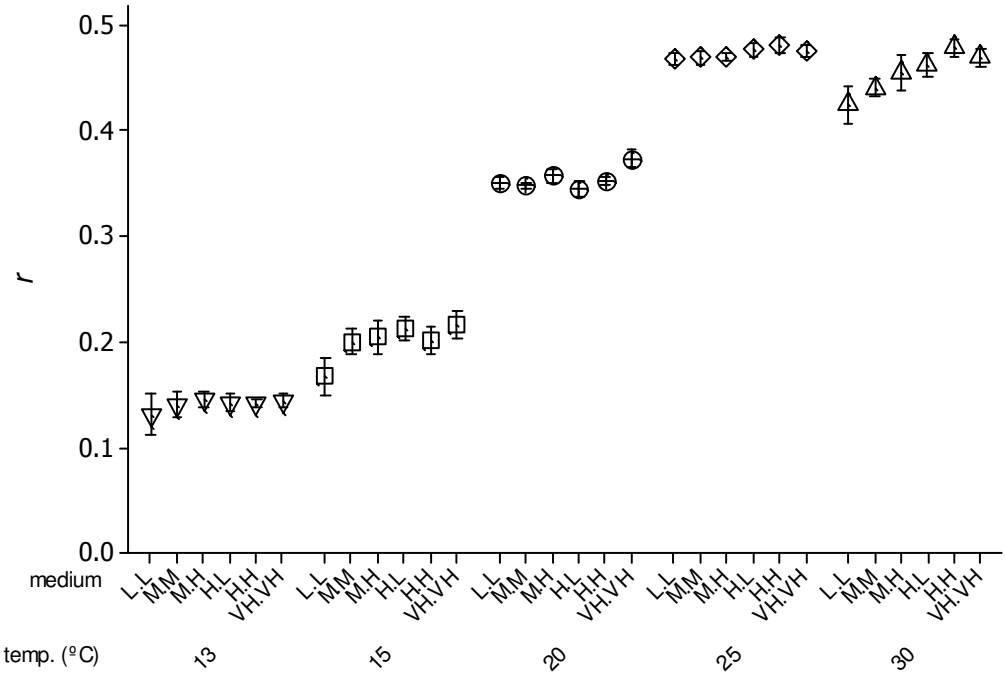

Figure 4.8: Combined effects of temperature and water chemistry on intrinsic rate of population growth (*r*) of *D. magna*. Symbols represent means and vertical bars represent 95% confidence intervals. Results of multiple comparisons among media are presented in Table 4.5 (Supplementary material).

4.4. Discussion

Being an ectothermic species, *D. magna* was affected by water temperature. This is evident in this study since all the studied endpoints were significantly affected by temperature, evidencing the crucial role of temperature on the life-history traits of *D. magna*. This is not surprising and has been reported by other authors (Goss and Bunting 1980; Goss and Bunting 1983; Heugens, Tokkie et al. 2006). Water chemistry also affected growth, reproduction and population growth of *D. magna*, although to a lesser extent than temperature. The importance of water chemistry on the life-history traits of this crustacean was been shown in previous work (Cowgill and Milazzo 1990; Cowgill and Milazzo 1991b). So, the major novelty of this study is on the interactive effects of temperature and water chemistry on the life-history traits of *D. magna*. We showed that the effects of water chemistry to *D. magna* are dependent on water temperature: major effects of water chemistry were observed at 20ºC and extreme temperatures (13 and 30ºC), whereas at 15 and 25ºC, the water chemistry caused variability only in a few endpoints.

The response of daphnids to water chemistry was more pronounced at 20ºC, probably due to the fact that they had been cultured at 20ºC for several years, and only a short acclimation period to other temperatures was allowed. Temperature acclimation plays a key role in the effects of high temperatures to organisms, in particular *Daphnia* (Paul, Lamkemeyer et al. 2004). Warm-acclimated *Daphnia* may have improved capacity to survive heat, as, for instance, due to higher quantities of heat-shock proteins (Feder and Hofmann 1999). Studies on the time course of temperature acclimation of *Daphnia* showed that the principal processes are completed in 2 weeks, but major changes occur in the first 2–5 days (Zeis, Maurer et al. 2004; Seidl, Pirow et al. 2005). In this study, parental daphnids were reared at the test temperature for at least 2 instars before the test neonates were released. At the highest temperature, 30ºC, this corresponds to about 3.5 days, thus, only sufficient time to allow for major acclimation processes. Hence, the procedure and duration of the temperature acclimation may have contributed to possible divergences between the findings of this study and previous studies.

Growth and development

Growth was mainly affected by temperature, with water chemistry causing only minor variability. At the 21st day, daphnids reared at 30ºC were smaller than the others, which follows the "temperature-size rule" and is concordant with previous studies (Chopelet, Blier et al. 2008). This is likely due to the reduction of the energy available for growth and reproduction, as explained below.

High temperatures (25 and 30ºC), increase Ca demand of daphnids because all physiological processes, including those involved in molting, are accelerated (Ashforth and Yan 2008). This is likely the reason why at these temperatures medium L.L caused reduced BL at day 21. The reduction in body length might have contributed to the reduced cumulative fertility observed in daphnids reared in this medium. Media H.H and VH.VH, those with higher hardness and alkalinity, caused the opposite effects.

In opposition, at temperatures equal or less than 20ºC we did not observe clear evidence of reduced growth and/or reproduction in medium L.L, as Ca demand was lower due to increasing intermoult intervals.

The somatic growth of daphnids between days 0 and 21 days was described based only on temperature, which presents evidence of the reduced role of water chemistry on daphnids growth within this water chemistry range. The growth model also illustrates that *D. magna* grow to bigger final sizes when reared at lower temperatures, which is concordant with the "temperature-size rule".

The number of juvenile instars was affected by temperature, with increasing numbers occurring at extreme temperatures (Goss and Bunting 1983), which is supported by the results of the present study. Water chemistry also affected the number of juvenile instars, most clearly at 20ºC, which is probably associated with variability of BL at day 0 (Green 1956) and water chemistry (as shown in Chapter 2).

Reproduction

The reproduction of *Daphnia* was also highly dependent on water temperature. *Daphnia* matured later and at a larger size at lower temperatures, which is in accordance with the "temperature-size rule" and with previous studies (McKee and Ebert 1996; Giebelhausen and Lampert 2001; Ashforth and Yan 2008). Thus, FFR would be expected to be higher at

lower temperatures, but this was not the case, showing that, besides SFR, also temperature affects the size of first brood (FFR), most notably at 13ºC. This is concordant with the evidence that the relationship body length – brood size is affected by temperature.

Although daphnids started reproducing earlier at 30ºC they did not produce more offspring at this temperature. In general, reproduction was higher at 25ºC, with extreme temperatures causing lower reproduction (Goss and Bunting 1983). This "bell shaped" response is likely related to the decreased feeding rates (Koh, Hallam et al. 1997) and other processes at extreme temperatures. At high temperatures, besides reduced feeding rate daphnids also face higher costs of maintenance, namely with the synthesis of stress proteins. In other hand, at low temperatures, daphnids face reduced ventilation and muscular activity (Zeis, Maurer et al. 2004), which reduces energy uptake forcing trade-offs. Thus, at extreme temperatures less energy is available for growth and reproduction, which causes impaired growth and/or reproduction.

At 30ºC *D. magna* seemed to be under severe stress: organisms hardly grew and the brood sizes were smaller than the expected at 20 or 25ºC, as illustrated by the lower slope of the relationship body length - brood size. The reduced brood size is related to the decreased instar duration and also to the subsequent increase of molting costs. Previous studies also pointed the temperature 30ºC to cause severe stress to *D. magna* (e.g. Goss and Bunting 1983) threatening *Daphnia* populations (Koh, Hallam et al. 1997).

A decrease in the cumulative fertility of *D. magna* at high temperature was also reported by Heugens and coworkers (2006), which they attributed to the reduced body length of daphnids. However, brood size depends not only on body length, but also on temperature: adult daphnids produced smaller broods at 30ºC than at 20ºC (Figure 4.4), thus contributing to reduced cumulative fertility at 30ºC.

At low temperatures daphnids showed reduced reproduction during the 21-d period, which was expected since they started reproducing later and have a longer intermoult period. Additionally, the reduced slope of the relationship body length - brood size might have contributed to the reduced reproduction: daphnids with the same size produced smaller broods at 15ºC than at 20ºC; however, given that only two broods were produced at 15ºC we have to interpret our results with precaution.

The production of non-viable offspring was most evident in all media at 13ºC and also in medium L.L at 30ºC. Abortion and degeneration of eggs and embryos in *Daphnia* species have been attributed to nutritional deficiencies, low food concentrations or transitory anoxia (Hall 1964). In our study, the production of non-viable offspring was most likely due to nutritional deficiencies (low Ca concentration in medium L.L) and/or reduced energy uptake due to reduced feeding rates. Anoxia was less likely to occur and, if occurred would have affected all daphnids at 30ºC and not only those in medium L.L.

Intrinsic rate of population growth

The intrinsic rate of population growth (*r*) of *D. magna* was positively related to temperature within the range 13-25ºC, above which a slight inversion was found. These results are consistent with results obtained in previous studies (Heugens, Tokkie et al. 2006; Ashforth and Yan 2008). However, although higher temperatures seem beneficial for the *Daphnia* population due to increased *r*, this might not be the case in natural ecosystems, as higher temperatures reduce the longevity of organisms. In fact, an inverse relationship exists between growth and longevity: rapid growth, as induced by higher temperatures, is associated with a correspondingly early senescence; and lower temperatures lead to slow rates of growth and ageing (Vijverberg 1989). According to Koh and coworkers (1997) it is common that populations, within their viable temperature range, reach high density in cold waters, consistently to the observations of Pratt (1943). This author observed that population size of *D. magna* at 18ºC was 2.5 times as great as that at 25ºC. Note that we determined *r* in a 21d experiment, not enough time to observe senescence of daphnids at high temperatures or cumulative reproduction at low temperatures, what might explain the differences between our findings and the findings reported by Pratt (1943).

Besides the evident effect of temperature, *r* is also affected by water chemistry: media with low hardness and alkalinity tend to reduce the population growth, which is probably related to the trend for increased AFR caused by these media.

Although the high variability of *r*, all values were positive, showing that populations of this clone of *D. magna* are likely to persist within the temperature range 13-30ºC, thus, agreeing with Koh and coworkers (1997), who reported that *Daphnia* populations can persist up to 30.6ºC, and reflects the eurythermy of this cladoceran.

Our results showed that the effects of water hardness and alkalinity to *Daphnia* are dependent on temperature, as they were more pronounced at 20ºC and extreme temperatures (13 and 30ºC), and less pronounced at 15 and 25ºC. We also found that high temperatures increased daphnids susceptibility to low hardness and alkalinity, concordantly to the findings of Ashforth and Yan (2008) for *D. pulex*. Furthermore, this interaction may become more significant under low food levels, as suggested by Ashforth and Yan (2008). In aquatic ecosystems, daphnids may be exposed to these conditions (low hardness and alkalinity and high temperature, together with low food levels) for long periods, with adverse consequences for population growth.

Temperatures equal or above 25ºC will cause severe stress to daphnids that will affect *Daphnia* populations. At these temperatures an increase in daphnids susceptibility to media with low hardness and alkalinity was observed, which can have cumulative effects at long-term (not visible in a 21d study). Similarly, a decrease in temperature below 15ºC will likely have negative effects for *Daphnia* population. However, under field conditions *Daphnia* populations may exhibit adaptation mechanisms to varying temperature, such as physiological strains adapted to different temperature regimes (Mitchell and Lampert 2000) as well as seasonal-succession of *Daphnia* clones (Carvalho and Crisp 1987).

Effects on the population growth of *Daphnia* might have serious repercussions in aquatic food webs, due to the key role that *Daphnia* play on food chains in aquatic ecosystems.

The effects described for *D. magna* might also be valid for other aquatic crustaceans. Like *Daphnia*, crustaceans are ectotherms and have large Ca demand, thus are particularly susceptible to changes in temperature and water chemistry.

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Supplementary material

Table 4.5 shows the mean values of life-history traits of *D. magna* at different temperatures.

Table 4.5: Mean values (standard error of the mean in parenthesis) of life-history traits of *D. magna* at different temperatures (N=120 for all variables except BL variables where N=60). Values with different letters are significantly different among temperatures. At each temperature, significant differences among test media are also shown (n≥10).

	Temperature				
	13ºC	15ºC	20ºC	25ºC	30ºC
BL at day 0 (mm)	0.90 (0.010) [a]	0.88 (0.009) [a]	0.84 (0.008) [b]	0.84 (0.007) [b]	0.74 (0.006) [c]
	---	---	---	---	---
BL at day 7 (mm)	2.33 (0.018) [a]	2.30 (0.025) [a]	2.96 (0.022) [b]	2.94 (0.013) [c]	2.47 (0.018) [d]
	H.L≠L.L	---	H.H=VH.VH≠M.M=L.L=M.H	---	---
BL at day 14 (mm)	3.43 (0.017) [a]	3.30 (0.012) [b]	3.65 (0.011) [c]	3.58 (0.017) [d]	2.98 (0.010) [e]
	H.L≠M.M	---	H.H≠M.H	L.L=M.M≠VH.VH	---
BL at day 21 (mm)	3.81 (0.015) [a]	3.79 (0.012) [a]	4.08 (0.010) [b]	3.90 (0.018) [c]	3.09 (0.010) [d]
	H.L≠M.H=M.M=VH.VH	M.M=M.H≠VH.VH	---	L.L≠M.H=H.H=VH.VH	L.L=M.M≠H.H
number of juvenile instars	5.00 (0.000) [a]	4.98 (0.012) [a]	4.58 (0.042) [b]	4.00 (0.000) [c]	4.20 (0.040) [d]
	---	---	VH.VH=H.H≠H.L=M.H=L.L=M.M	---	VH.VH=H.H=M.M=M.H≠L.L
adult instars duration	5.81 (0.033) [a]	4.88 (0.035) [b]	3.13 (0.019) [c]	2.38 (0.009) [d]	1.95 (0.012) [e]
	---	---	---	---	---
AFR (d)	18.85 (0.039) [a]	15.91 (0.111) [b]	9.39 (0.068) [c]	6.43 (0.024) [d]	5.93 (0.039) [e]
	---	---	VH.VH≠H.H≠M.H=M.M=L.L	---	---
SFR (mm)	3.43 (0.017) [a]	3.28 (0.010) [b]	3.01 (0.022) [c]	2.45 (0.012) [d]	2.19 (0.017) [e]
	H.L≠M.H=M.M	---	VH.VH=H.H≠M.H	---	L.L≠M.H=VH.VH
FFR	14.77 (0.435) [a]	21.05 (0.240) [b]	16.65 (0.443) [c]	11.47 (0.165) [d]	8.09 (0.180) [e]
	L.L≠M.M=H.H=H.L=VH.VH=M.H	---	H.H=VH.VH=H.L≠M.M=L.L=M.H	---	---
cumulative fertility	14.75 (0.44) [a]	35.92 (1.27) [b]	107.50 (1.19) [c]	173.91 (1.85) [d]	95.53 (1.51) [e]
	---	---	L.L=M.M=M.H=H.H≠H.L	L.L≠ H.H	L.L≠ H.L=H.H
number of broods	1.00 (0.000) [a]	1.63 (0.047) [b]	4.42 (0.049) [c]	6.34 (0.044) [d]	7.96 (0.030) [e]

	---	---	L.L=M.M=M.H≠H.H≠VH.VH	---	---

Table 4.5 (cont.)

	Temperature				
	13ºC	15ºC	20ºC	25ºC	30ºC
average brood size	14.75 (0.44) [a]	21.60 (0.28) [b]	25.30 (0.19) [c]	27.39 (0.19) [d]	11.98 (0.18) [e]
	L.L≠M.M=H.H=H.L=VH.VH=M.H	---	H.H≠H.L	---	---
non-viable offspring	3.38 (0.290) [a]	0.00 (0.000) [b]	0.04 (0.04) [b]	0.027 (0.013) [b]	0.12 (0.025) [b]
	H.H=H.L≠VH.VH≠M.M≠L.L	---	---	---	VH.VH=H.H=M.M=H.L=M.H≠L.L
intrinsic rate of population growth, r	0.14 (0.002) [a]	0.20 (0.003) [b]	0.35 (0.001) [c]	0.47 (0.001) [d]	0.45 (0.003) [e]
	---	L.L≠M.M=H.H=M.H=H.L=VH.VH	H.L≠VH.VH	---	L.L≠VH.VH=H.H

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CHAPTER

5

EFFECTS OF HARDNESS AND ALKALINITY ON COPPER AND ZINC TOXICITY TO *DAPHNIA MAGNA*: CONSEQUENCES FOR SURVIVAL AND FEEDING

5. Effects of hardness and alkalinity on copper and zinc toxicity to *Daphnia magna*: consequences for survival and feeding

Abstract

In this paper we studied the effects of hardness and alkalinity on the acute and sublethal toxicity (feeding inhibition) of Cu and Zn to *Daphnia magna* in order to answer the following questions: Can the acute toxicity of Cu and Zn to *D. magna* be described simply as a function of water hardness and alkalinity? Are the acute toxicity values found for *D. magna* accurately predicted by the biotic ligand model (BLM)? Can the sublethal toxicity (feeding inhibition) of Cu and Zn to *D. magna* be described as a function of water hardness and alkalinity? Are the effects of hardness and alkalinity on the toxicity of Cu and Zn similar for both acute and sublethal toxicity? We observed that decreasing hardness raised the acute toxicity of Zn, whereas decreasing alkalinity raised the acute toxicity of Cu and the sublethal toxicity of copper and decreased the sublethal toxicity of zinc, showing that hardness might not be the major water chemistry parameter controlling metals toxicity. The BLM could not accurately predict the metals acute toxicity, as it underpredicted Zn acute toxicity and overpredicted Cu acute toxicity. Moreover, we showed that hardness and alkalinity could be used as chemical parameters to describe both acute and sublethal toxicity of Cu and Zn to *D. magna*.

Keywords: Cu; Zn; *Daphnia magna*; hardness; alkalinity; acute toxicity; feeding inhibition

5.1. Introduction

The aquatic toxicity of metals is a widespread and current environmental problem. Metals are continuously released to the environment not only by anthropogenic processes, but also by natural processes, namely erosion of underlying and local rocks and volcanic activity (Garrett 2000). Moreover, metals do not break down, but are persistent in the environment. Cu and Zn are naturally occurring metals commonly present in surface waters. In surface waters located near mining areas, these metals can reach high concentrations, for instance

higher than 15.74 µM Cu and 76.48 µM Zn (Milu, Leroy et al. 2002; Smolders, Lock et al. 2003).

The toxicity of metals to aquatic organisms can be predicted based on the water chemistry parameters. For instance, USEPA aquatic life criteria for copper used to be based on water hardness (USEPA 2007). However, in 2007 USEPA updated the criteria for protection of aquatic life in ambient freshwater by incorporating the biotic ligand model (BLM), a metal bioavailability model (USEPA 2007). This model considers the contribution of several water chemistry parameters for the prediction of metals toxicity, including the type and concentration of dissolved organic carbon (DOC), alkalinity, pH and concentration of major ions. However, analyzing all these water chemistry parameters may be cost-demanding and difficult to manage from a regulatory point of view. Thus, we hypothesized that Cu and Zn toxicity could be described based on the integrative parameters hardness and alkalinity.

The majority of the BLM studies focus either on the acute or chronic toxicity of metals to aquatic organisms. However, the effect of water chemistry on the sublethal toxicity (feeding activity) remains quite unknown. Thus, we assessed the effects of hardness and alkalinity on the feeding of *D. magna* exposed to Cu or Zn. The feeding activity of *D. magna* constitutes a sensitive indicator of toxicity and is an ecologically relevant endpoint (McWilliam and Baird 2002). Food intake determines energy acquisition and further allocation, thus affecting developmental rate, growth rate, fecundity, and survival of individuals (Nogueira, Baird et al. 2004), thus determining population structure and dynamics. The establishment of functional relationships between water chemistry, metals and the feeding activity of *D. magna* is essential to provide a better and more realistic understanding of potential long-term effects of metals to cladocerans.

Therefore, the objectives of the present study are to answer the following questions: Can the acute toxicity of Cu and Zn to *D. magna* be described simply as a function of water hardness and alkalinity? Are the acute toxicity values found for *D. magna* accurately predicted by the BLM? Can the sublethal toxicity (feeding inhibition) of Cu and Zn to *D. magna* be described as a function of water hardness and alkalinity? Are the effects of hardness and alkalinity on the toxicity of Cu and Zn similar for both acute and sublethal toxicity?

To achieve these goals we carried out several acute and sublethal toxicity tests with *D. magna* exposed to Cu or Zn. This allowed us to develop simple models to describe both acute and sublethal toxicity of these metals as a function of hardness and alkalinity. We also assessed the accuracy of the BLM-predictions under the water chemistry scenarios used in this study by comparing BLM-predictions with our experimental results.

5.2. **Material and Methods**

5.2.1. Experimental organisms

Daphnia magna Straus (1820) clone F (sensu Baird, Barber et al. 1990) were cultured in ASTM hard water (ASTM 2004) enriched with a standard organic additive (Marinure seaweed extract, supplied by Glenside Organics Ltd.) and fed *Pseudokirchneriella subcapitata* (5.5 µg dw/ml/day). Culture medium was renewed every other day. Temperature was 20 ± 1ºC and photoperiod was 16 h light: 8 h dark.

Parental daphnids briefly acclimated to the test parameters of hardness and alkalinity. Egg carrying females, 7-8 days old, were transferred to each test medium and were cultured in that test medium keeping the other conditions as described above. Neonates from the first and second broods were discarded, and neonates from third-to-fifth broods of these females were used in the experiments, since the latter had been exposed to the test media during their entire developmental period (Barata, Baird et al. 2007).

5.2.2. Experimental design

Thirteen synthetic media, with varying hardness and alkalinity, were tested. Hardness varied between 0.47 and 3.57mM as $CaCO_3$ (7.6-fold); alkalinity varied between 0.32 and 2.31mM as $CaCO_3$ (7.2-fold). The selection of these hardness and alkalinity ranges had in consideration two aspects: the variability of these parameters occurring in natural surface waters, and the chemical conditions essential for daphnids, avoiding, for instance, very soft waters. We also tested media in which hardness and alkalinity were not correlated, which allowed the assessment of the effects of hardness and alkalinity independently of each other.

Hardness and alkalinity of the test media were manipulated through the addition of different volumes of stock solutions of $CaSO_4.2H_2O$, $MgSO_4.7H_2O$, $NaHCO_3$ and KCl (analytical-grade) to ultrapure water.

The toxicity of both Cu and Zn was tested in each test media. Cu was added as $CuCl_2.2H_2O$ (Merck) and Zn as $ZnCl_2$ (Riedel de-Haën) and were reagents of analytical grade. Stock solutions of these metals were used to prepare concentration series of each metal. In order to obtain near-equilibrium situations, all media were stored at 20ºC for 15-24h prior to the beginning of the experiments (Heijerick, De Schamphelaere et al. 2002).

5.2.3. Acute immobilization

Acute immobilization tests were performed with juvenile *D. magna* (<24 h old), exposed to Cu or Zn at 20ºC and photoperiod 16 h light: 8 h dark, and followed the recommendations of the OECD Test Guideline 202 (OECD 2004). Tests consisted of at least six treatments (control + five metal concentrations) and each treatment consisted of 5 replicates with 5 daphnids in each replicate. The number of immobilized daphnids in each replicate was recorded after 48 h.

5.2.4. Feeding inhibition

Feeding inhibition tests were performed with fourth instar daphnids (4 days old) which had been cultured in the test medium. Tests were carried out in vials containing 50 ml of the test medium and the desired metal concentration (including a control) and the algae *P. subcapitata* at the concentration 5.5 µg dw/ml. Each treatment consisted of five replicates (having three daphnids each) and four blanks (having no daphnids). The vials were kept 6 hours in the dark at 20 ±1ºC. These test conditions were shown to elicit a significant feeding of the daphnids, preventing both settlement of the algae and starvation of the daphnids (unpublished data). After the exposure period daphnids were carefully removed and their body length was later estimated based on the length of the first exopodite of the second antennae, as described previously (Chapter 2). The vials were vigorously shaken before the measurement of the optical density in a spectrophotometer (Jenway 6505 Spectrophotometer ultraviolet-visible) at 440 nm. Feeding rates were calculated using the

equation by Allen and coworkers (Allen, Calow et al. 1995), with slight adaptations as shown in equation 5.1:

$$F = \frac{V(C_0 - C_t)}{tN} \qquad \text{(equation 5.1)}$$

where:

F = feeding rate of single animals (µg dw/ind/h)

V = volume of suspension (ml)

C_0 = cell concentration in the vials without daphnids (µg dw/ml)

C_t = final cell concentration in the treatment (µg dw/ml)

t = time animals were allowed to feed (h)

N = number of animals per replicate

5.2.5. Chemical measurements

Chemical analyses were performed to quantify the concentration of metals, concentration of major cations, alkalinity, hardness, conductivity, temperature and pH at the end of each test. Samples for analyses of dissolved Cu, Zn, Ca, Mg, Na and K were filtered (0.45 µm, cellulose acetate) and acidified to pH<2 with HNO_3 before analysis using a Inductively Coupled Plasma - Atomic Emission Spectrometer (Perkin-Elmer Optima 5300 ICP-AES). In the feeding inhibition tests, since metals can adsorb to the algae surface (Wilde, Stauber et al. 2006), decreasing the concentration of dissolved metal, we also determined total Cu and Zn (non-filtered samples).

Total hardness and total alkalinity were determined by the EDTA and the bromocresol green titrimetric procedures, respectively (American Public Health Association 2005). Conductivity was determined using a WTW Cond 330i meter; temperature and pH were determined using a WTW pH 330 meter. In the feeding inhibition tests, the concentration of dissolved organic carbon (DOC) was measured in random samples. Samples were filtered with a 0.45 µm polypropylene membrane and then analyzed using a Sievers 800 Portable TOC analyzer (Ionics-Sievers. Instruments, Inc., CO, USA).

5.2.6. Data treatment and statistics

Results of the acute and feeding inhibition tests were used to determine EC_{50} (50% effect concentrations) and associated 95% confidence limits by adjusting the results to a second order decay model (McWilliam and Baird 2002).

For acute immobilization tests, EC_{50} values were determined for the 48h exposure period and were based on dissolved metal concentrations. The acute toxicity of Zn was not determined in media with high values of hardness (above 2.15 mM) and simultaneous high values of alkalinity (above 1.74 mM) due to chemical precipitation of Zn which reduced the dissolved fraction of Zn and impeded the occurrence of high mortality rates.

In feeding inhibition tests, EC_{50} values were calculated on the basis of total and dissolved metal concentrations. As for the acute tests, the precipitation of Zn solids prevented the determination of EC_{50} feeding inhibition values (fraction: dissolved Zn) for media with alkalinity above 1.72 mM. Under these conditions algae settled out, preventing daphnids feeding.

To assess whether acute and sublethal toxicity of the metals could be described simply as a function of the parameters hardness and alkalinity, we performed linear regressions with the statistical package Minitab© 15 (Minitab Inc., USA).

5.2.7. Comparison of BLM predictions with the experimental results

The BLM (Windows Interface, Version 2.2.3, downloaded from http://www.hydroqual.com/wr_blm.html) was used to predict acute 48h-LC_{50} of both Cu and Zn to *D. magna*. Since the BLM requires a minimum DOC concentration and % HA (humic acids) to work properly, we assumed that: DOC concentration = 0.05 mg/L and % HA = 10. The concentration of sulfate and chloride was estimated based on measurements carried out under similar conditions in previous studies (Chapter 2): sulfate concentration was estimated based on the sum of Ca and Mg concentrations (linear regression, r^2=0.999; n=10); and chloride was estimated based on K concentration (linear regression, r^2=0.951; n=10).

Since BLM predicts toxicity values as free metal ion, we further used it in the speciation mode to determine the corresponding dissolved metal concentration and, thus, allowing comparison to the toxicity values we determined in this work. However, it is important to

highlight that BLM predictions refer to mortality (LC_{50}), whereas our results refer to immobilization (EC_{50}).

5.3. **Results**

The results of the chemical analyses to the test media are presented in Table 5.1.

In this study we did not intend to manipulate each ion independently, but to manipulate hardness and alkalinity. Thus, statistical correlations between some of the water quality parameters were found, as shown in Table 5.2. Hardness and alkalinity were not correlated (p=0.511). Hardness was correlated to Ca, Mg and conductivity, whereas alkalinity was correlated to Na and H.

Table 5.1: Chemical parameters of the test media, n≥ 6. In the feeding inhibition tests the DOC concentration was 0.55mg/L

	hardness	alkalinity		Conductivity	Ionic concentration, mM			
medium	(mM $CaCO_3$)	(mM $CaCO_3$)	pH	(µS/cm)	Ca	Mg	Na	K
1	0.47	0.62	8.33	205.7	0.17	0.24	1.08	0.07
2	0.92	2.31	8.75	589.5	0.31	0.50	4.18	0.20
3	0.92	1.72	8.71	502.8	0.34	0.49	3.37	0.16
4	1.34	0.59	8.30	375.6	0.54	0.78	1.07	0.09
5	1.81	1.14	8.30	564.3	0.71	0.97	2.24	0.11
6	1.81	0.32	8.06	404.5	0.71	1.00	0.56	0.10
7	1.84	1.17	8.52	552.6	0.67	0.98	2.18	0.10
8	2.17	2.20	8.65	797.2	0.85	1.22	4.45	0.20
9	2.69	0.32	8.04	549.7	1.07	1.51	0.56	0.17
10	3.11	0.60	8.27	686.8	1.16	1.65	1.11	0.17
11	3.13	1.18	8.48	795.5	1.20	1.70	2.20	0.19
12	3.57	1.75	8.63	937.5	1.33	2.40	3.26	0.14
13	3.56	0.32	8.00	695.5	1.36	1.96	0.55	0.21

Table 5.2: Pearson correlation coefficients for relationships between water quality parameters. Numbers in bold text denote coefficients that are significant at $p<0.05$ (n=13)

	Ca	K	Mg	Na	H	conductivity	alkalinity
K	0.502						
Mg	**0.978**	0.461					
Na	-0.224	0.361	-0.126				
H	0.367	-0.0376	0.256	**-0.855**			
conductivity	**0.762**	**0.701**	**0.808**	0.455	-0.251		
alkalinity	-0.235	0.361	-0.13	**0.997**	**-0.862**	0.448	
hardness	**0.998**	0.514	**0.983**	-0.193	0.326	**0.784**	-0.201

5.3.1. Acute immobilization

The individual effects of hardness and alkalinity on the acute toxicity of Cu and Zn (dissolved fraction) to *D. magna*, are depicted in Figures 5.1 and 5.2; the regression models of acute and sublethal toxicity of both metals to *D. magna* are presented in Table 5.3.

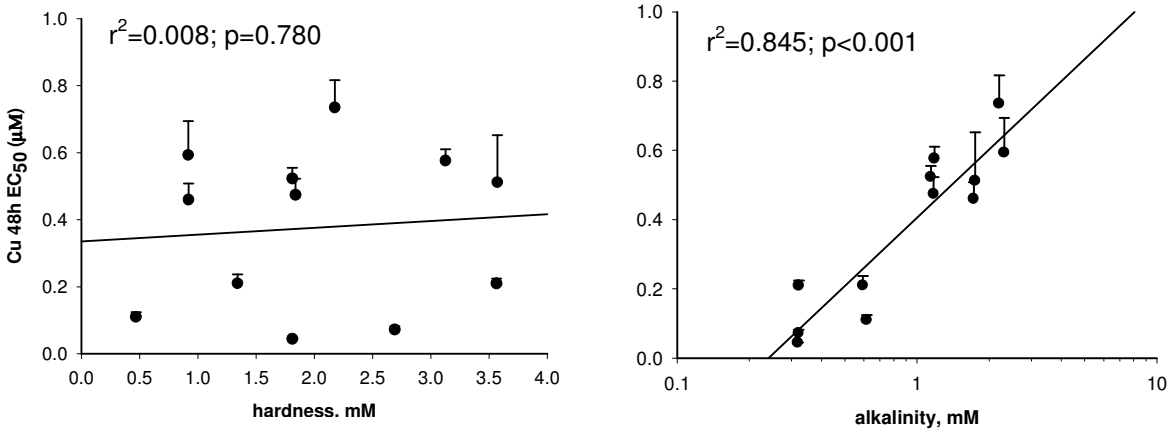

Figure 5.1: Acute Cu toxicity to *D. magna* (48-h EC_{50} values in µM + 95% confidence intervals) as a function of hardness (left panel); and alkalinity (right panel). Both hardness and alkalinity are expressed as mM as $CaCO_3$. Linear regressions are noted as solid lines.

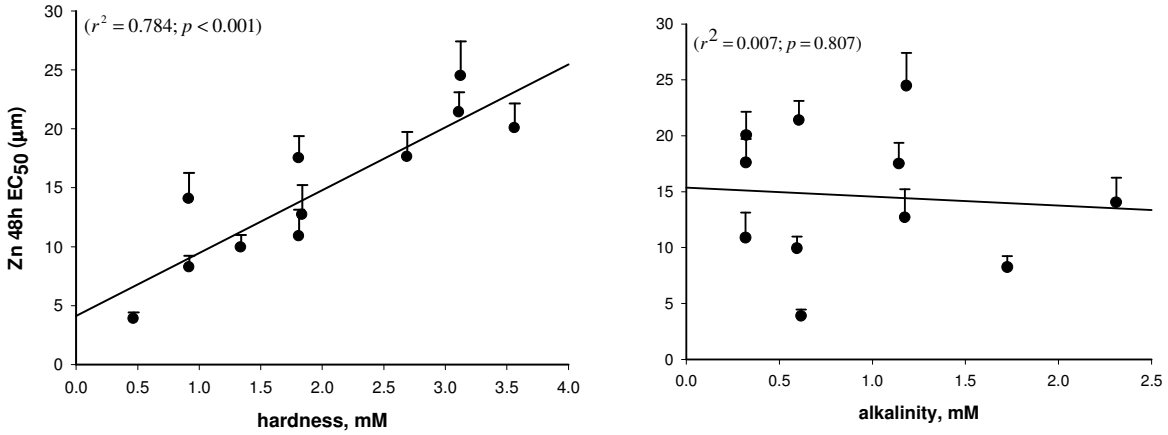

Figure 5.2: Acute Zn toxicity to *D. magna* (48-h EC_{50} values in µM + 95% confidence intervals) as a function of hardness (left panel) and alkalinity (right panel). Both hardness and alkalinity are expressed as mM as $CaCO_3$. Linear regressions are noted as solid lines.

Over all the acute experimental treatments, Cu 48h EC_{50}s varied 17-fold and Zn 48h EC_{50}s varied 6-fold. The acute Cu toxicity to *D. magna* was mainly determined by alkalinity, which explained 84.5% of the variability around the 48h EC_{50} values (logarithmic relationship). Although hardness was not significant *per se*, including this parameter in the regression increased the model fit (adj-r^2 increased from 82.9% to 91.6%).

In contrast to Cu, acute Zn toxicity was mainly controlled by hardness, which explained 78.4% of the variability (linear relationship). Although alkalinity *per se* was not significant (p=0.807), it contributed to improve the models' fit, as the adj-r^2 increased from 76.0% (only hardness) to 90.1% (both hardness and alkalinity; Table 5.3). Thus, linear regressions concerning acute toxicity of Cu and Zn to *D. magna* were highly significant, with adjusted coefficients of regression (adj-r^2) above 0.9.

Table 5.3: Regression models for acute and sublethal toxicity of Cu and Zn as a function of hardness and/or alkalinity. No evidence of lack of fit (P >0.05) for any of the models

acute toxicity (immobilization)	Cu	Coefficient	Std Error	p	overall regression
	Constant	0.345	0.0266	<0.001	F(2, 9)=61.22
	log (hardness, mM)	0.258	0.0766	0.008	r2-adj=0.916
	log (alkalinity, mM)	0.688	0.0629	<0.001	p<0.001
	Zn	Coefficient	Std Error	p	overall regression
	Constant	2.928	1.355	0.063	F(2, 8)=46.52
	hardness, mM	6.479	0.674	<0.001	r2-adj=0.901
	log (alkalinity, mM)	8.538	2.298	0.006	p<0.001
sublethal toxicity (feeding inhibition)	Cu, total	Coefficient	Std Error	p	overall regression
	constant	-0.383	0.0919	0.002	F(2, 10)=67.70
	hardness, mM	0.141	0.0301	<0.001	r2-adj=0.917
	alkalinity, mM	0.501	0.0441	<0.001	p<0.001
	Zn, total	Coefficient	Std Error	p	overall regression
	constant	25.789	3.659	<0.001	F(2, 9)=19.44
	log (hardness, mM)	34.977	9.533	0.005	r2-adj=0.770
	log (alkalinity, mM)	-39.714	8.251	<0.001	p<0.001
	Cu, dissolved	Coefficient	Std Error	p	overall regression
	constant	-0.0451	0.0667	0.515	F(1, 10)=36.79
	alkalinity, mM	0.303	0.05	<0.001	r2-adj=0.765
					p<0.001
	Zn, dissolved	Coefficient	Std Error	p	overall regression
	constant	28.05	4.183	<0.001	F(1, 8)=14.818
	log (alkalinity, mM)	-52.301	13.587	0.005	r2-adj=0.606
					p=0.005

5.3.2. Feeding inhibition

The individual effects of hardness and alkalinity on the sublethal toxicity of Cu and Zn to *D. magna*, considering the total concentration and dissolved fraction of these metals, are illustrated in Figures 5.3 and 5.4. Over all the feeding experimental treatments, Cu 6h EC_{50}s varied 19-fold and Zn 6h EC_{50}s varied 5-fold as total metal, whereas Cu 6h EC_{50}s varied 27-fold and Zn 6h EC_{50}s varied 4-fold as dissolved metal.

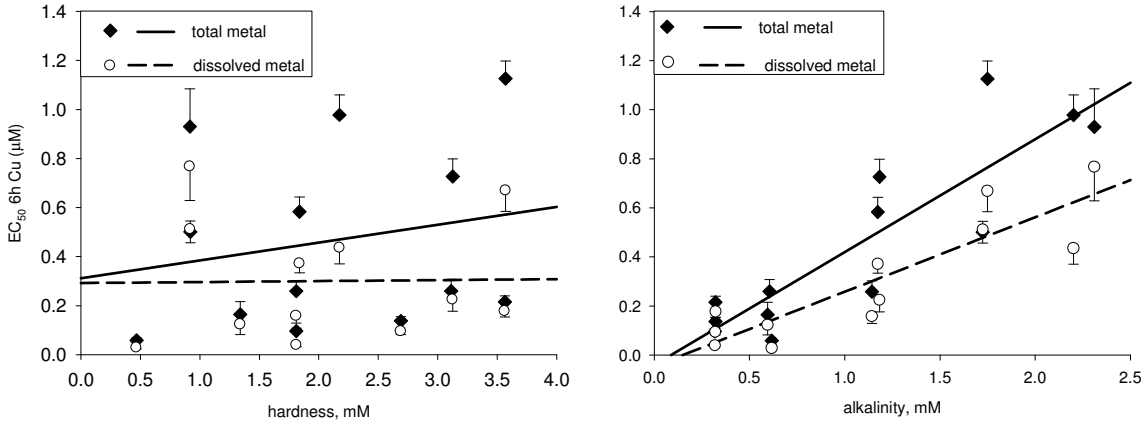

Figure 5.3: Sublethal Cu toxicity (6-h EC_{50} feeding inhibition) to *D. magna* as a function of hardness (left panel; r^2 total = 0.042; p>0.05; r^2 dissolved = 0.000; p>0.05); and alkalinity (right panel; r^2 total = 0.779; p<0.001; r^2 dissolved = 0.786; p<0.001). Both hardness and alkalinity are expressed as mM as $CaCO_3$. Error bars represent 95% confidence intervals; linear regressions are noted as lines.

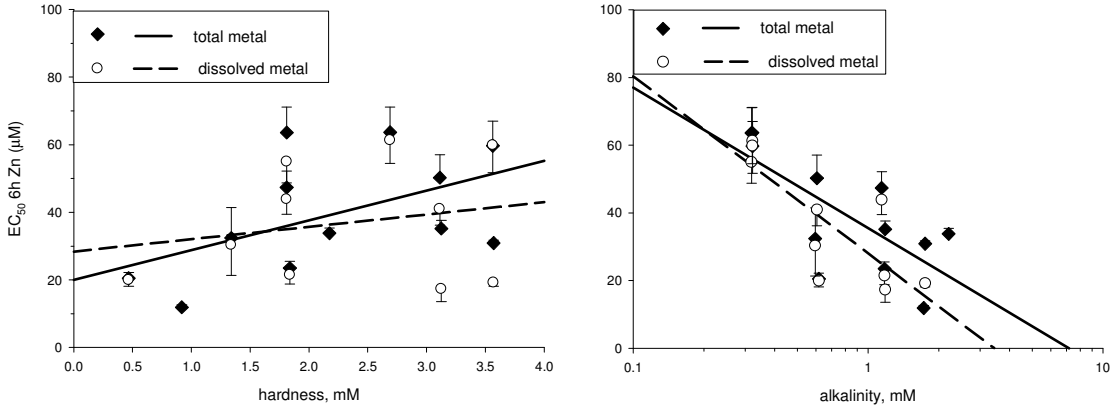

Figure 5.4: Sublethal Zn toxicity (6-h EC_{50} feeding inhibition) to *D. magna* as a function of hardness (left panel; r^2 total = 0.270; p>0.05; r^2 dissolved = 0.046; p>0.05); and alkalinity (right panel; r^2 total = 0.531; p<0.001; r^2 dissolved = 0.649; p<0.001). Both hardness and alkalinity are expressed as mM as $CaCO_3$. Error bars represent 95% confidence intervals; linear regressions are noted as lines.

The sublethal toxicity of both metals was influenced by alkalinity, although in opposite ways: rising alkalinity decreased Cu toxicity but increased Zn toxicity.

The sublethal Cu toxicity (total and dissolved) to *D. magna* varied similarly to the acute Cu toxicity: it decreased with increasing alkalinity and was not affected by hardness. However, the relationship between alkalinity and sublethal Cu toxicity was linear, whereas the relationship between alkalinity and acute Cu toxicity was logarithmic. Although not significant *per se*, hardness contributed significantly to improve the model fit relative to total metal by increasing the adj-r^2 from 75.9% (only alkalinity) to 91.7%.

The sublethal Zn toxicity increased with increasing alkalinity, and was not influenced by hardness.

All linear regression equations were statistically significant, showing higher adjustment to the experimental results for the total metals and, in particular, for Cu.

5.3.3. Comparison of BLM predictions with the experimental results

Measured vs predicted acute toxicity values for Cu and Zn are shown in Figure 5.5. BLM underpredicted acute Zn toxicity (i.e., $LC_{50's}$ are higher than EC_{50}'s) for all water chemistry scenarios: the BLM-predicted values are 1.8 to 6.6 fold higher to the observed values. In opposition, BLM overpredicted Cu acute toxicity (i.e., $LC_{50's}$ are lower than EC_{50}'s) in particular for media with higher alkalinity. The observed EC_{50} values are 0.9 to 6.4-fold higher to the BLM-predicted toxicity values. On the other hand, the models developed in the present study (Table 3) could predict the EC_{50} values with a factor of less than 1.6 for Cu and 1.3 for Zn. It should, however, be recognized that this model is a descriptive model since it was especially developed to fit the measured EC_{50} values.

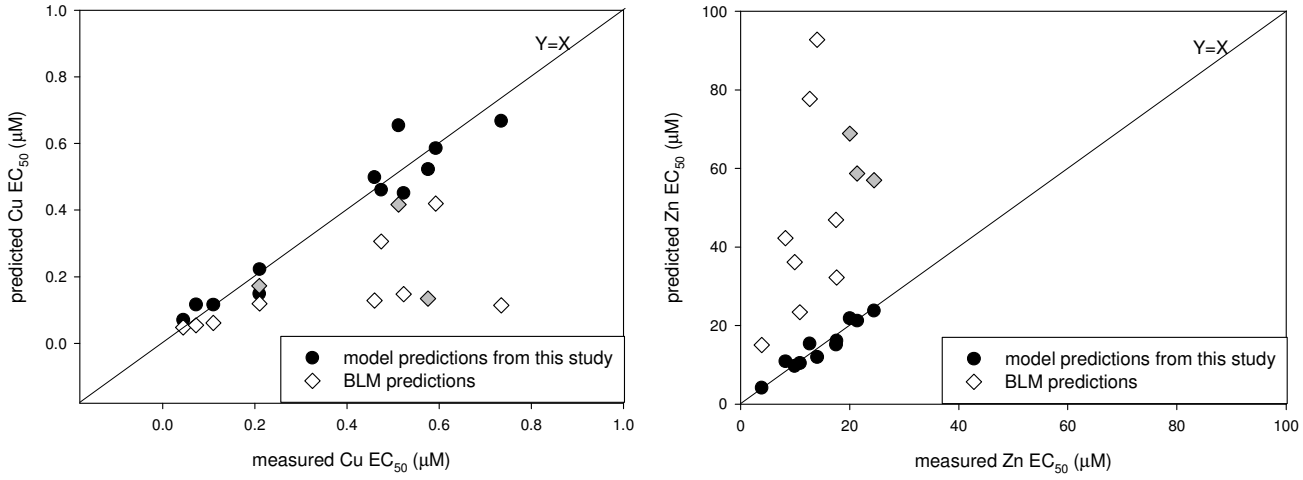

Figure 5.5: Relationship between measured and predicted acute toxicity for Cu (left panel) and Zn (right panel). Circles refer to the predictions of the descriptive model from this study (Table 5.3); diamonds refer to BLM-predicted 48h LC_{50} values and the gray fill denotes media with sulfate concentration exceeding 2.90 mM - the upper bound to which the BLM has been calibrated. The line represents Y=X, i.e., predicted values = measured values.

5.4. Discussion

In this paper we studied the effects of varying hardness and alkalinity on the acute toxicity (immobilization) and sublethal toxicity (feeding inhibition) of both Cu and Zn to *D. magna*. Despite the fact that hardness and alkalinity varied about 7-fold (7.6 and 7.2-fold, for hardness and alkalinity respectively), Cu toxicity varied more widely (17 to 27-fold) and Zn toxicity varied less widely (4 to 6-fold). This reflects the higher degree of sensitivity to water chemistry of Cu toxicity compared to Zn. Within this water chemistry range, hardness and alkalinity allowed a good description of the toxicity of both metals, in particular acute toxicity. However, although we attributed the variability of metals toxicity to hardness and alkalinity, we should bear in mind that hardness and alkalinity are, actually, integrative parameters reflecting ionic concentration, as illustrated by the correlation coefficients in Table 5.2.

The effects of water chemistry on acute and sublethal toxicity varied according to the metal considered: acute and sublethal toxicity of Cu was determined by alkalinity, whereas Zn toxicity was determined by hardness or alkalinity, respectively in acute and sublethal tests. This is in line with the findings of previous studies which reported that acute Cu toxicity was less sensitive to hardness than Zn (Yim, Kim et al. 2006).

Acute Cu toxicity was not affected by water hardness. This contrasts with the general idea that increased hardness decreases Cu toxicity (through competition), as was assumed, for instance, by the USEPA for development of aquatic life criteria for Cu (USEPA 2007). However, other studies also reported no effect of hardness on Cu toxicity to aquatic organisms (De Schamphelaere and Janssen 2004c; Hyne, Pablo et al. 2005; Markich, Batley et al. 2005). Alkalinity, on the other hand, had a significant effect on Cu toxicity. In the past, alkalinity was not considered in a regulatory context probably because hardness and alkalinity are usually strongly correlated in most natural waters. However, in the present study the lack of correlation between hardness and alkalinity makes clear a strong relationship between alkalinity and Cu toxicity. The protective effect of alkalinity against Cu acute toxicity to aquatic crustaceans (Gensemer, Naddy et al. 2002; Hyne, Pablo et al. 2005) is related to complexation of Cu by higher concentrations of hydroxide and/or carbonate (USEPA 2007), with a subsequent reduced metal bioavailability (Hyne, Pablo et al. 2005). At high pH, hydroxy complexes ($CuOH^+$) and carbonate ($CuCO_3$) can also be bioavailable, but they are less bioavailable than Cu^{2+} (De Schamphelaere, Heijerick et al. 2002). However, the protective effect of alkalinity may be confounded with the protective effect of Na, since these two parameters are commonly correlated, as in the present study. Increasing Na concentration decreases Cu toxicity to aquatic crustaceans (De Schamphelaere and Janssen 2002a; Gensemer, Naddy et al. 2002; Ryan, Tomasso et al. 2009) probably due to increased Na^+ competition with Cu for binding at the site of action (De Schamphelaere and Janssen 2002a; De Schamphelaere and Janssen 2004c).

Hardness had a protective effect on Zn toxicity (e.g. Paulauskis and Winner 1988; Heijerick, De Schamphelaere et al. 2005; Hyne, Pablo et al. 2005), which is attributed to the competitive interaction between Ca^{2+} and Zn for binding sites at the site of action which reduces Zn uptake (Komjarova and Blust 2009) and also to the effect of the water hardness on the general health of these organisms (Heijerick, Janssen et al. 2003; Muyssen, De

Schamphelaere et al. 2006). On the other hand, acute Zn toxicity was not significantly affected by alkalinity (Figure 5.2), pH or Na concentration (data not shown) which agrees with a previous study (Clifford and McGeer 2009).

The sublethal toxicity of both metals was determined by alkalinity. Concerning Cu, previous studies showed that chronic toxicity to *Daphnia* is not or only slightly affected by water hardness (Winner 1985; De Schamphelaere and Janssen 2004c) but significantly reduced by increasing pH or Na (De Schamphelaere and Janssen 2004c). The fact that hardness and alkalinity affect both acute and sublethal toxicity similarly suggests that the same mechanisms might be involved in both acute and sublethal Cu toxicity to *D. magna*, and agrees with a previous study which reported that dietary Cu exposure was irrelevant to chronic toxicity (De Schamphelaere and Janssen 2004b).

Sublethal Zn toxicity increased with alkalinity. This supports a previous study which found that increasing pH (correlated to increased alkalinity) increased the chronic Zn toxicity to *D. magna* (Heijerick, De Schamphelaere et al. 2005), which the authors attributed to reduced competition effect between H^+ and Zn^{2+}. However, we suggest that the increased chronic Zn toxicity with increasing pH might be related to toxicity via dietary route. Since increasing pH (correlated to increasing alkalinity) increases Zn absorption to algae cell walls (Harrison, Campbell et al. 1986), daphnids might be uptaking more Zn (through ingestion) than occurred only through water, which explains increased toxicity compared to acute exposures. This represents an important indication that mechanisms that determine acute and chronic Zn toxicity for *D. magna* may differ. Similarly to Heijerick and coworkers (2005), who found a logarithmic relationship between pH and chronic Zn toxicity to *D. magna*, we found a logarithmic relationship between alkalinity and sublethal Zn toxicity.

The simple models we presented have a high descriptive capacity for acute and sublethal toxicity of both Cu and Zn to *D. magna* within this water chemistry range (adj-r^2> 0.90, except sublethal Zn toxicity, which adj-r^2 was 0.77). Thus, although they may constitute a preliminary predicting tool in future studies, they should not be used as effective predictive tools until further validation. Note that we did not intend to develop a predictive model for Cu and Zn toxicity but, rather, to assess if the integrative parameters hardness and alkalinity could be used to describe acute and sublethal toxicity of Cu and Zn.

The use of mathematical models to predict metals toxicity in natural waters requires prudence. First, application of the models is limited to the range of water chemistry parameters used to develop them (De Schamphelaere and Janssen 2004c). Second, natural waters can be chemically very complex, causing divergences between the results of toxicity tests in natural waters and concurrent laboratory waters. This is supported by Van Genderen and coworkers (2007) who found a significant relationship between alkalinity and Cu toxicity in laboratory waters but no relationship in natural waters.

There is one aspect of water hardness which was not addressed in this study: the ratio Ca:Mg. In this study the ratio Ca:Mg was kept reasonably constant (mean ± SD: 0.69±0.049 on a molar basis, n=13), whereas in natural waters this ratio can vary widely. The ratio Ca:Mg has been pointed out to be more important than the absolute concentrations of both ions. However, the effect of the ratio Ca:Mg seems to be variable, depending on several aspects, namely the test organism, the hardness level and the metal. Thus, although the developed models could describe acute and sublethal toxicity of Cu and Zn to *D. magna*, further refinement and validation with a broader range of exposure chemical conditions and field collected water samples should be performed.

Among the models developed for sublethal toxicity of each metal (total and dissolved fraction) we observed that the total metal concentration was better correlated to water chemistry than the dissolved fraction. This is in accordance with other studies which report that total Cu concentration might be a better predictor of biological response (i.e. toxicity) than free Cu^{2+} activity, due to competition effects or the bioavailability of inorganic and organic copper complexes (De Schamphelaere and Janssen 2002a). In addition, the dietary route (metal adsorbed to algae which is measured as total metal but not as dissolved or free ion metal) might be contributing to the metals toxicity.

Concerning BLM-predictions, we found that BLM could not accurately predict Cu and Zn acute toxicity, which might be due to the following aspects. First, we used no source of DOC but assumed 0.05mg DOC/L and 10% HA (humic acids) to run the BLM. Note that the BLM does not run for DOC concentrations lower than 0.05 mg/L. However, if we were indeed subestimating or overestimating the complexation capacity of the DOC, similar results would have been obtained for both metals, which was not the case, since BLM

underpredicted Zn toxicity but overpredicted Cu toxicity. Second, the genetic variability among *D. magna* clones: we used the clone F (sensu Baird, Barber et al. 1990) whereas the BLM was calibrated with several clones. It is known that acute and sublethal sensitivity to metals is affected by genetic clonal variability (Baird, Barber et al. 1990). The fact that BLM could not accurately predict metals acute toxicity, presents evidence that the model might not be suitable for all water chemistry scenarios and/or *D. magna* clones.

Acknowledgments

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CHAPTER

6

GENERAL DISCUSSION

6. **General discussion**

In this thesis we assessed the ecological importance of the combined variation of the main water physicochemical parameters (hardness, alkalinity, pH and temperature) to the life-history traits of crustaceans, either in unpolluted and metal polluted conditions. To represent crustaceans we used *Daphnia magna*. This species is one of the most commonly used test organism in ecological and ecotoxicological studies. *Daphnia* occupy a central position in food webs, since they are often the primary grazers of phytoplankton and the primary forage for planktivorous fish. *Daphnia* are mainly found in small water bodies (Hebert 1978; Lampert 1991) which represents an advantage for this study because these systems are more susceptible to varying water chemistry since chemical variations are not buffered by the presence of a large water mass (Gordo, Lubian et al. 1994). As other crustaceans, *Daphnia* are ectothermic and have large Ca demand (Jeziorski and Yan 2006). For these reasons *Daphnia* was appropriate for the purpose of this study.

Studying the effects of varying water physicochemical parameters to aquatic biota is pertinent since all aquatic systems are potentially exposed to changes in the water parameters, which are a consequence of environmental phenomena such as acid deposition and climate change. Although the effects of the variation of single parameters to aquatic crustaceans have been studied, little is known about the combined effects of varying water physicochemical parameters simultaneously, which is, indeed, what occurs in natural ecosystems.

The relative importance of water physicochemical parameters to *Daphnia* is schematically illustrated in Figure 6.1. Although the importance of hardness to *Daphnia* is known, we showed that the effects of temperature and pH overrule the effects of hardness that, in turn, overrule the effects of alkalinity.

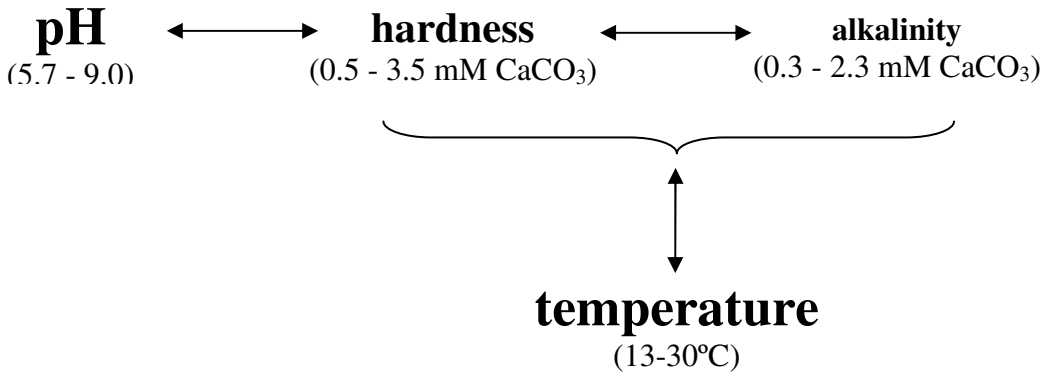

Figure 6.1: Schematic representation of the relative importance of water physicochemical parameters to *D. magna*, concerning the effects on growth, reproduction and intrinsic rate of population growth. Arrows represent interaction or potential interaction (see text for details) and increased font size represents increased effect on growth, reproduction and intrinsic rate of population growth of *D. magna*.

The findings of this thesis are relevant for understanding and anticipating the effects of varying water physicochemical parameters to aquatic biota, particularly crustaceans. As referred previously, all aquatic ecosystems are exposed to varying water physicochemical parameters namely hardness, alkalinity, pH and temperature. In aquatic ecosystems hardness is being reduced in consequence of decreased input of Ca and Mg, which is due to alterations in the adjacent soils (forest biomass harvesting and regrowth after multiple timber harvesting cycles), as well as acid deposition (Ashforth and Yan 2008; Jeziorski, Yan et al. 2008). Hardness is a parameter with a key importance to crustaceans, particularly to *Daphnia*, due to their large Ca demand (Ashforth and Yan 2008). For instance, growth and reproduction of *Daphnia* were impaired by low hardness, mediated by low Ca concentration (similarly to Cowgill and Milazzo 1991c; Hessen, Alstad et al. 2000). However, the magnitude of the effects of hardness on the reproduction of *Daphnia* appeared to be dependent on the alkalinity level. In fact, we showed that "alkalinity also matters", although to a less extent than hardness. Besides the suggested interaction with hardness on reproduction, it significantly affected the intrinsic rate of population growth. Moreover, it might have more relevant effects on other crustaceans species, such as *Ceriodaphnia*, which are more sensitive to alkalinity than *Daphnia* (Cowgill and Milazzo 1991b).

Aquatic systems may also experience water acidification, mainly due to the natural soil acidification process accelerated by acid deposition (Skjelkvåle, Stoddard et al. 2005), thus

being exposed to simultaneous variation of pH and hardness. Following our results, the effects of pH are more pronounced that the effects of hardness. *Daphnia* are very susceptible to low pH due to their finely balanced Na metabolism (Glover and Wood 2005). Low pH reduced the survival, growth, reproduction, feeding rate and intrinsic rate of population growth of *Daphnia*, concordantly to previous studies (Kring and O'Brien 1976; Alibone and Fair 1981; Walton, Compton et al. 1982; Havas 1985; Locke and Sprules 2000; Weber and Pirow 2009). There seemed to be an interaction between pH and hardness: low hardness enhanced the susceptibility of *Daphnia* to low pH, which agrees with previous studies (Hooper, Connon et al. 2008). This interaction can play an important role in aquatic ecosystems, in particular concerning the extinction of *Daphnia* populations. Although the extinction of *Daphnia* populations has been attributed to decreasing Ca concentration (Jeziorski, Yan et al. 2008), it might be, indeed, a consequence of decreasing both Ca concentration and pH, as supported by the decreased survival of daphnids at low hardness and simultaneously low pH.

Moreover, due to the phenomenon of global warming the water temperature in aquatic ecosystems is expected to increase and, thus, ecosystems will be exposed to the simultaneous variation of temperature and water chemistry parameters. Under these circumstances, the effects of temperature will probably overrule the effects of water hardness and alkalinity, as supported by the fact that temperature affected all the studied endpoints. In general, extreme temperatures reduced growth, reproduction and, thus, intrinsic rate of population growth of *Daphnia* (similarly to Goss and Bunting 1983; Mourelatos and Lacroix 1990; Koh, Hallam et al. 1997; Chopelet, Blier et al. 2008). The effects of extreme temperatures on reproduction were partly due to the reduced slopes of the relationship body length - brood size. Additionally, high temperatures enhanced the susceptibility of *Daphnia* to low hardness (concordantly to Ashforth and Yan 2008).

Putting our results together, we conclude that *Daphnia* living in soft waters will be particularly vulnerable to the effects of high temperature and decreasing pH, which is in line with previous studies (Ashforth and Yan 2008; Hooper, Connon et al. 2008). This combination of physicochemical parameters represents a scenario likely to occur and, thus, a reason for concern in natural water bodies.

Soft water systems are those at higher potential risk from acid deposition (Lonergan and Rasmussen 1996) and declining Ca concentration (Jeziorski, Yan et al. 2008) and, as we have shown, also those in which low pH and increasing temperature will cause more adverse effects. This combination of low hardness, low pH and high temperature can have serious effects not only to *Daphnia* but also to other acid-sensitive and Ca demanding species. In soft waters (hardness 0.5 mM as $CaCO_3$ in this study) *Daphnia* will not survive at pH 5.7 and they will suffer reduced growth, reproduction and, thus, reduced population growth at pH 6.7. Moreover, increasing temperature will enhance the adverse effects of low hardness: in soft waters *Daphnia* showed reduced growth and cumulative fertility at 25ºC, and additional adverse effects on reproduction and population growth of *Daphnia* were observed at 30ºC.

To emphasize the importance of water physicochemical parameters to *Daphnia* we can also refer to their effects on endpoints which are rarely studied. For instance, we observed that both hardness and alkalinity affected the number of juvenile instars. Furthermore, we also observed that both hardness and temperature affected the relationship body length - brood size: generally *Daphnia* at low hardness or extreme temperatures produced smaller broods than daphnids in other conditions, i.e, low hardness and extreme temperatures reduced the reproductive success of *Daphnia*. Additionally, pH and hardness affected the feeding rate of both juvenile and adult daphnids. Thus, it is unequivocal that water physicochemical parameters are determinant for *Daphnia* reproduction.

However, in field conditions *Daphnia* populations may exhibit adaptation mechanisms to the variation of water physicochemical parameters, namely temperature. Among the adaptation mechanisms there are acclimation (Paul, Lamkemeyer et al. 2004), physiological strains adapted to various temperature regimes (Mitchell and Lampert 2000) and also seasonal-succession of *Daphnia* clones (Carvalho and Crisp 1987). On the other hand, they will be exposed to other stressors, which can exacerbate or mitigate the effects of the physicochemical parameters. For instance, *Daphnia* will be more susceptible to low hardness and alkalinity at low food levels (Ashforth and Yan 2008).

The effects described for *D. magna* are, most likely, valid for other aquatic crustaceans. Like *Daphnia*, crustaceans are ectotherms and have large Ca demand and, thus are particularly susceptible to changes in temperature and water hardness. Thus, water

physicochemical changes will threaten not only the survival and performance of aquatic crustaceans but also, indirectly, other species by affecting the structure of aquatic food webs.

The consequences of the hardness-mediated amelioration of effects are important in laboratory assays since most assays are conducted using hard water (Locke 1991), following recommendations from international organizations (e.g. OECD 2008). At this hardness levels, both pH and temperature effects are less pronounced that at lower hardness. Thus, some species may be more sensitive to pH or temperature than suggested by bioassays conducted in hard water. Thus, it is recommended that future studies on the impact of physicochemical parameters in aquatic ecosystems should have in consideration the effects of simultaneous variation of several parameters for two reasons. Firstly, in natural waters chemical parameters are usually correlated (Okland and Okland 1986; Jeziorski, Yan et al. 2008), thus, from an ecological point of view, it makes sense to study their combined effects rather than their individual effects. Secondly, studying the effects of single parameters might lead to an incorrect estimation of the real effects, as interactions among environmental parameters affect biota in ways that cannot be predicted based on their individual effects (Christensen, Graham et al. 2006).

Water hardness and alkalinity also affected acute and sublethal toxicity of Cu and Zn to *D. magna*, as depicted in Figure 6.2. The effects on feeding rate are particularly important since to the best of our knowledge, this topic was not studied previously.

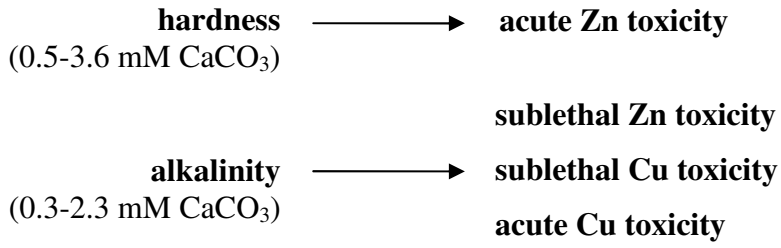

Figure 6.2: Schematic representation of the effects of water hardness and alkalinity on Zn and Cu toxicity to *D. magna*. Acute toxicity was determined as immobilization and sublethal toxicity was determined as feeding inhibition

Although hardness protected *Daphnia* against the adverse effects of pH and temperature it did not protect *Daphnia* against both metals, only Zn. Increasing hardness ameliorated acute Zn toxicity to *Daphnia* (similarly to Paulauskis and Winner 1988; Heijerick, De Schamphelaere et al. 2005; Hyne, Pablo et al. 2005). Water hardness was considered the main water chemistry parameter affecting metals toxicity, namely Cu (USEPA 2007), which was related to the strong correlation with alkalinity in most natural waters. This study supports previous studies stating that hardness may not be the major water chemistry parameter controlling metals toxicity to aquatic organisms, as is the case of Cu (De Schamphelaere and Janssen 2004c; Hyne, Pablo et al. 2005; Markich, Batley et al. 2005).
Furthermore, the important effect of alkalinity in the toxicity of metals to *Daphnia* contrasts with its small (although significant) effect in the absence of metals. Increasing alkalinity reduced Cu acute and sublethal toxicity to daphnids (concordantly to Gensemer, Naddy et al. 2002; Hyne, Pablo et al. 2005) but increased sublethal Zn toxicity (in line with the findings of Heijerick, De Schamphelaere et al. 2005).
Within the tested water chemistry range, hardness and alkalinity allowed a good description of both acute and sublethal toxicity of Cu and Zn to *D. magna*. However, the models were developed to fit the results and, thus, require further validation. The use of mathematical models to predict metals toxicity in natural waters requires prudence because it is only valid within a certain water chemistry range and because of the huge complexity of natural waters. The fact that BLM could not accurately predict the metals acute toxicity, gives an important indication that it might not be appropriate for all water chemistry scenarios and/or *D. magna* clones.

In this thesis we showed that water physicochemical parameters hardness, alkalinity, pH and temperature have combined effects on growth, reproduction and intrinsic rate of population growth of *Daphnia*. Other parameters, such as survival and feeding rate may be also affected, both in the presence and absence of metals. *Daphnia* living in soft waters are more susceptible to the effects low pH, increasing temperature and metals toxicity, which raises concern about the effects of acid deposition, global warming and metal pollution on aquatic ecosystems. This thesis highlights the importance of water physicochemical parameters to *Daphnia*, agreeing with the findings of Steiner (2004) who reported that

Daphnia biomass in fishless ponds was strongly related to water physicochemical parameters (pH and temperature), but had no relationship with biotic factors (predators abundance and algal resource production or quality). Water physicochemical parameters might play an important role to other aquatic crustaceans, thus, affecting the structure, stability and functioning of aquatic ecosystems.

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